IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION:

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FOR:

NON-ENDOGENOUS, CONSTITUTIVELY ACTIVATED KNOWN G PROTEIN-COUPLED RECEPTORS

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NON-ENDOGENOUS, CONSTITUTIVELY ACTIVATED KNOWN G PROTEIN-COUPLED RECEPTORS

This patent application claims priority from U.S. Provisional Application Number 60/195,747, filed with the United States Patent and Trademark Office on April 7, 2000; and is related to U.S. Serial Number 09/170,496, filed with the United States Patent and Trademark Office on October 13, 1998, each of which is incorporated in its entirety by reference.

FIELD OF THE INVENTION

The invention disclosed in this patent document relates to transmembrane receptors, and more particularly to G protein-coupled receptors for which the endogenous ligand has been identified ("known GPCR"), and specifically to known GPCRs that have been altered to establish or enhance constitutive activity of the receptor. Most preferably, the altered GPCRs are used for the direct identification of candidate compounds as receptor agonists, inverse agonists or partial agonists for use as therapeutic agents.

BACKGROUND OF THE INVENTION

Although a number of receptor classes exist in humans, by far the most abundant and therapeutically relevant is represented by the G protein-coupled receptor (GPCR or GPCRs) class. It is estimated that there are some 100,000 genes within the human genome, and of these, approximately 2%, or 2,000 genes, are estimated to code for GPCRs. Receptors, including GPCRs, for which the endogenous ligand has been identified are referred to as "known" receptors, while receptors for which the endogenous ligand has not been identified are referred to as "orphan" receptors. GPCRs represent an important area

for the development of pharmaceutical products: from approximately 20 of the 100 known GPCRs, 60% of all prescription pharmaceuticals have been developed.

GPCRs share a common structural motif. (All these receptors have seven sequences of between 22 to 24 hydrophobic amino acids that form seven alpha helices, each of which spans the membrane (each span is identified by number, *i.e.,* transmembrane-1 (TM-1), transmebrane-2 (TM-2), etc.). The transmembrane helices are joined by strands of amino acids between transmembrane-2 and transmembrane-3, transmembrane-4 and transmembrane-5, and transmembrane-6 and transmembrane-7 on the exterior, or "extracellular" side, of the cell membrane (these are referred to as "extracellular" regions 1, 2 and 3 (EC-1, EC-2 and EC-3), respectively). The transmembrane helices are also joined by strands of amino acids between transmembrane-1 and transmembrane-2, transmembrane-3 and transmembrane-4, and transmembrane-5 and transmembrane-6 on the interior, or "intracellular" side, of the cell membrane (these are referred to as "intracellular" regions 1, 2 and 3 (IC-1, IC-2 and IC-3), respectively). The "carboxy" ("C") terminus of the receptor lies in the intracellular space within the cell, and the "amino" ("N") terminus of the receptor lies in the extracellular space outside of the cell.

Generally, when an endogenous ligand binds with the receptor (often referred to as "activation" of the receptor), there is a change in the conformation of the intracellular region that allows for coupling between the intracellular region and an intracellular "G-protein." It has been reported that GPCRs are "promiscuous" with respect to G proteins, *i.e.,* that a GPCR can interact with more than one G protein. *See*, Kenakin, T., 43 *Life Sciences* 1095 (1988). Although other G proteins exist, currently, Gq, Gs, Gi, Gz and Go are G proteins that have been identified. Endogenous ligand-activated GPCR coupling with the G-protein begins a signaling cascade process (referred to as "signal transduction"). Under normal

conditions, signal transduction ultimately results in cellular activation or cellular inhibition. It is thought that the IC-3 loop as well as the carboxy terminus of the receptor interact with the G protein.

Under physiological conditions, GPCRs exist in the cell membrane in equilibrium between two different conformations: an "inactive" state and an "active" state. A receptor in an inactive state is unable to link to the intracellular signaling transduction pathway to produce a biological response. Changing the receptor conformation to the active state allows linkage to the transduction pathway (via the G-protein) and produces a biological response.

A receptor may be stabilized in an active state by an endogenous ligand or a compound such as a drug. Recent discoveries, including but not exclusively limited to modifications to the amino acid sequence of the receptor, provide means other than endogenous ligands or drugs to promote and stabilize the receptor in the active state conformation. These means effectively stabilize the receptor in an active state by simulating the effect of an endogenous ligand binding to the receptor. Stabilization by such ligand-independent means is termed "constitutive receptor activation."

Traditional ligand-dependent screens seek to indirectly identify compounds that antagonize the action of the ligand on the receptor in an effort to prevent ligand-induced activation of the receptor. However, such compounds, sometimes referred to as neutral-antagonists, generally would not be expected to affect the ligand-independent activity, or overactivity, of the receptor and the subsequent abnormal cellular response that can result from this overactivity. This is particularly relevant to a growing number of diseases, such as those identified in the table below, that have been linked to overactive GPCRs, because traditional neutral-antagonists will not block the abnormal ligand-independent activity of these receptors.

Background Table 1

Disease	Overactive GPCR
Schizophrenia	5-HT2A, D2
Depression	5-HT2A
Hyperthyroidism	Thyrotropin
Hypertension	Angiotensin AT1A
Asthma	Adenosine A1
Melanoma	MC-1
Retinitis Pigmentosa	Rhodopsin receptor

SUMMARY OF THE INVENTION

Disclosed herein are non-endogenous versions of endogenous, known GPCRs and uses thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides graphic results of comparative analysis of a co-transfection of non-endogenous TSHR-A623I ("signal enhancer") and an endogenous target receptor, in this case GPR24 ("GPR24 wt"), versus non-endogenous, constitutively activated versions of the target receptor GPR24 ("T255K," "T255K/T257R," "24-IC3-SST2," "C305Y,"

"P271L," "W269C," "W269F," "W269L," "F265I," I261Q," and "D140N") co-transfected with non-endogenous TSHR-A623I, utilizing an adenylyl cyclase assay. This assay involved the addition of TSH and MCH, the endogenous ligands for TSHR and GPR24, respectively.

Figure 2 provides graphic results of comparative analysis of a co-transfection of non-endogenous signal enhancer TSHR-A623I (with and without TSH) and endogenous target receptor GPR5 ("GPR5 wt"), versus non-endogenous, constitutively activated target receptor GPR5 ("V224K") co-transfected with non-endogenous TSHR-A623I (with and without TSH), utilizing an adenylyl cyclase assay.

Figures 3A-3E provide a diagrammatic representation of the signal measured comparing CMV, non-endogenous, constitutively activated GPCRs, utilizing 8XCRE-Luc reporter plasmid.

Figure 4 provides an illustration of IP_3 production from several non-endogenous versions of GPR24 as compared with the endogenous version of this receptor.

Figure 5 is a graphic representation of the results of a membrane-based cyclic AMP assay providing comparative results for constitutive signaling of TSHR-A623K:Fusion Protein and a control vector (pCMV).

DETAILED DESCRIPTION

The scientific literature that has evolved around receptors has adopted a number of terms to refer to ligands having various effects on receptors. For clarity and consistency, the following definitions will be used throughout this patent document. To the extent that these definitions conflict with other definitions for these terms, the following definitions shall control:

AGONISTS shall mean materials (*e.g.*, ligands, candidate compounds) that activate the intracellular response when they bind to the receptor, or enhance GTP binding to membranes.

AMINO ACID ABBREVIATIONS used herein are set out in Table A:

TABLE A		
ALANINE	ALA	A
ARGININE	ARG	R
ASPARAGINE	ASN	N
ASPARTIC ACID	ASP	D
CYSTEINE	CYS	C
GLUTAMIC ACID	GLU	E
GLUTAMINE	GLN	Q
GLYCINE	GLY	G
HISTIDINE	HIS	H
ISOLEUCINE	ILE	I
LEUCINE	LEU	L
LYSINE	LYS	K
METHIONINE	MET	M
PHENYLALANINE	PHE	F
PROLINE	PRO	P
SERINE	SER	S
THREONINE	THR	T
TRYPTOPHAN	TRP	W
TYROSINE	TYR	Y
VALINE	VAL	V

PARTIAL AGONISTS shall mean materials (*e.g.*, ligands, candidate compounds) that activate the intracellular response when they bind to the receptor to a lesser degree/extent than do agonists, or enhance GTP binding to membranes to a lesser degree/extent than do agonists.

ANTAGONIST shall mean materials (*e.g.*, ligands, candidate compounds) that competitively bind to the receptor at the same site as the agonists but which do not activate the intracellular response initiated by the active form of the receptor, and can thereby inhibit the intracellular responses by agonists or partial agonists. ANTAGONISTS do not diminish the baseline intracellular response in the absence of an agonist or partial agonist.

CANDIDATE COMPOUND, in the context of the disclosed invention, shall mean a small molecule (for example, and not limitation, a chemical compound) that is amenable to a screening technique.

COMPOSITION means a material comprising at least one component; a "pharmaceutical composition" is an example of a composition.

COMPOUND EFFICACY shall mean a measurement of the ability of a compound to inhibit or stimulate receptor functionality, as opposed to receptor binding affinity. Exemplary means of detecting compound efficacy are disclosed in the Example section of this patent document.

CODON shall mean a grouping of three nucleotides (or equivalents to nucleotides) which generally comprise a nucleoside (adenosine (A), guanosine (G), cytidine (C), uridine (U) and thymidine (T)) coupled to a phosphate group and which, when translated, encodes an amino acid.

CONSTITUTIVELY ACTIVATED RECEPTOR shall mean a receptor subject to constitutive receptor activation. A constitutively activated receptor can be endogenous or non-endogenous.

CONSTITUTIVE RECEPTOR ACTIVATION shall mean stabilization of a receptor in the active state by means other than binding of the receptor with its endogenous ligand or a chemical equivalent thereof.

CONTACT or **CONTACTING** shall mean bringing at least two moieties together, whether in an in vitro system or an in vivo system.

DIRECTLY IDENTIFYING or **DIRECTLY IDENTIFIED**, in relationship to the phrase "candidate compound", shall mean the screening of an candidate compound against a constitutively activated receptor, preferably a constitutively activated receptor, and most preferably against a constitutively activated G protein-coupled cell surface receptor, and assessing the compound efficacy of such compound. This phrase is, under no circumstances, to be interpreted or understood to be encompassed by or to encompass the phrase "indirectly identifying" or "indirectly identified."

ENDOGENOUS shall mean a material that a mammal naturally produces. ENDOGENOUS in reference to, for example and not limitation, the term "receptor," shall mean that which is naturally produced by a mammal (for example, and not limitation, a human) or a virus. By contrast, the term **NON-ENDOGENOUS** in this context shall mean that which is not naturally produced by a mammal (for example, and not limitation, a human) or a virus. For example, and not limitation, a receptor which is not constitutively active in its endogenous form, but when manipulated becomes constitutively active, is most preferably referred to herein as a "non-endogenous, constitutively activated receptor." Both terms can be utilized to describe both "in vivo" and "in vitro" systems. For example, and not limitation, in a screening approach, the endogenous or non-endogenous receptor may be in reference to an in vitro screening system. As a further example and not limitation, where the genome of a mammal has been manipulated to include a non-endogenous constitutively activated receptor, screening of a candidate compound by means of an in vivo system is viable.

G PROTEIN COUPLED RECEPTOR FUSION PROTEIN and **GPCR FUSION PROTEIN**, in the context of the invention disclosed herein, each mean a non-endogenous protein comprising an endogenous, constitutively activate GPCR or a non-endogenous, constitutively activated GPCR fused to at least one G protein, most preferably the alpha (α) subunit of such G protein (this being the subunit that binds GTP), with the G protein preferably being of the same type as the G protein that naturally couples with endogenous GPCR. For example, and not limitation, in an endogenous state, if the G protein "Gsα" is the predominate G protein that couples with the GPCR, a GPCR Fusion Protein based upon the specific GPCR would be a non-endogenous protein comprising the GPCR fused to Gsα; in some circumstances, as will be set forth below, a non-predominant G protein can be fused to the GPCR. The G protein can be fused directly to the c-terminus of the constitutively active GPCR or there may be spacers between the two.

HOST CELL shall mean a cell capable of having a Plasmid and/or Vector incorporated therein. In the case of a prokaryotic Host Cell, a Plasmid is typically replicated as a autonomous molecule as the Host Cell replicates (generally, the Plasmid is thereafter isolated for introduction into a eukaryotic Host Cell); in the case of a eukaryotic Host Cell, a Plasmid is integrated into the cellular DNA of the Host Cell such that when the eukaryotic Host Cell replicates, the Plasmid replicates. Preferably, for the purposes of the invention disclosed herein, the Host Cell is eukaryotic, more preferably, mammalian, and most preferably selected from the group consisting of Hek-293, Hek-293T and COS-7 cells.

INDIRECTLY IDENTIFYING or **INDIRECTLY IDENTIFIED** means the traditional approach to the drug discovery process involving identification of an endogenous ligand specific for an endogenous receptor, screening of candidate compounds against the receptor for determination of those which interfere and/or compete with the ligand-receptor

interaction, and assessing the efficacy of the compound for affecting at least one second messenger pathway associated with the activated receptor.

INHIBIT or **INHIBITING**, in relationship to the term "response" shall mean that a response is decreased or prevented in the presence of a compound as opposed to in the absence of the compound.

INVERSE AGONISTS shall mean materials (*e.g.*, ligand, candidate compounds) which bind to either the endogenous form of the receptor or to the constitutively activated form of the receptor, and which inhibit the baseline intracellular response initiated by the active form of the receptor below the normal base level of activity which is observed in the absence of agonists or partial agonists, or decrease GTP binding to membranes. Preferably, the baseline intracellular response is inhibited in the presence of the inverse agonist by at least 30%, more preferably by at least 50%, and most preferably by at least 75%, as compared with the baseline response in the absence of the inverse agonist.

KNOWN RECEPTOR shall mean an endogenous receptor for which the endogenous ligand specific for that receptor has been identified.

LIGAND shall mean an endogenous, naturally occurring molecule specific for an endogenous, naturally occurring receptor.

MUTANT or **MUTATION** in reference to an endogenous receptor's nucleic acid and/or amino acid sequence shall mean a specified change or changes to such endogenous sequences such that a mutated form of an endogenous, non-constitutively activated receptor evidences constitutive activation of the receptor. In terms of equivalents to specific sequences, a subsequent mutated form of a human receptor is considered to be equivalent to a first mutation of the human receptor if (a) the level of constitutive activation of the subsequent mutated form of a human receptor is substantially the same as that evidenced by

the first mutation of the receptor; and (b) the percent sequence (amino acid and/or nucleic acid) homology between the subsequent mutated form of the receptor and the first mutation of the receptor is at least about 80%, more preferably at least about 90% and most preferably at least 95%. Ideally, and owing to the fact that the most preferred cassettes disclosed herein for achieving constitutive activation includes a single amino acid and/or codon change between the endogenous and the non-endogenous forms of the GPCR, the percent sequence homology should be at least 98%.

NON-ORPHAN RECEPTOR shall mean an endogenous naturally occurring molecule specific for an endogenous naturally occurring ligand wherein the binding of a ligand to a receptor activates an intracellular signaling pathway.

ORPHAN RECEPTOR shall mean an endogenous receptor for which the endogenous ligand specific for that receptor has not been identified or is not known.

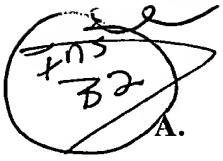
PHARMACEUTICAL COMPOSITION shall mean a composition comprising at least one active ingredient, whereby the composition is amenable to investigation for a specified, efficacious outcome in a mammal (for example, and not limitation, a human). Those of ordinary skill in the art will understand and appreciate the techniques appropriate for determining whether an active ingredient has a desired efficacious outcome based upon the needs of the artisan.

PLASMID shall mean the combination of a Vector and cDNA. Generally, a Plasmid is introduced into a Host Cell for the purposes of replication and/or expression of the cDNA as a protein.

STIMULATE or **STIMULATING**, in relationship to the term "response" shall mean that a response is increased in the presence of a compound as opposed to in the absence of the compound.

VECTOR in reference to cDNA shall mean a circular DNA capable of incorporating at least one cDNA and capable of incorporation into a Host Cell.

The order of the following sections is set forth for presentational efficiency and is not intended, nor should be construed, as a limitation on the disclosure or the claims to follow.



A. Introduction

Constitutively active forms of known G protein-coupled receptors, disclosed in the present patent document, can be obtained by site-directed mutational methods, well-known to those skilled in the art. A constitutively active receptor useful for direct identification of candidate compounds is preferably achieved by mutating the receptor at a specific location within an intracellular loop, most preferably within the intracellular loop three (IC3) region. Such mutation can produce a non-endogenous receptor that is intended to be constitutively activated, as evidenced by an increase in the functional activity of the receptor, for example, an increase in the level of second messenger activity. While standard methods of site-directed mutagenesis may be employed, a preferred method is one that is disclosed in a co-pending, commonly assigned patent document U.S. Application Serial No. 09/170,496, filed October 13, 1998, which is incorporated herein by reference.

Table B below lists known endogenous GPCRs that have been converted to non-endogenous versions thereof, their respective G protein and endogenous ligand.

TABLE B

Known GPCRs	G Protein	Endogenous Ligand
5HT-1A	Gi	Serotonin
5HT-1B	Gi	Serotonin

5HT-1D	Gi	Serotonin
5HT-1E	Gi	Serotonin
5HT-1F	Gi	Serotonin
5HT-2B	Gi	Serotonin
5HT-4A	N/I	Serotonin
5HT-4B	N/I	Serotonin
5HT-4C	N/I	Serotonin
5HT-4D	N/I	Serotonin
5HT-4E	N/I	Serotonin
5HT-5A	Unknown	Serotonin
5HT-6	Gs	Serotonin
5HT-7	Gs	Serotonin
AVPR1A	Gq	Arginine Vasopressin
AVPRIB	Gq	Arginine Vasopressin
AVPR2	Gs	Arginine Vasopressin
BBR3	Gq	Bombesin
BDKR1	Gq	Bradykinin
BDKR2	Gq	Bradykinin
C3a	N/I	Anaphylatoxin
C5a	N/I	Anaphylatoxin
CB1	Gi	Cannabinoid
CB2	Gi	Cannabinoid
CCR2b	Gi	Monocyte chemoattractant (MCP)
CCR3	Gi	Eotaxin, Leukotactin-1, RANTES, MCP
CCR5	Gi	MIP-1α, MIP-1β, RANTES
CCR8	Gi	I-309, TARC, MIP-1β
CCR9	N/I	Thymus-expressed chemokine (TECK)
CRFR1	Gs	Corticotropin-releasing-factor
CXCR4	Gi	SDF1
Dopamine D1	Gs	Dopamine
Dopamine D2	Gi	Dopamine
Dopamine D3	Gi	Dopamine
Dopamine D5	Gs	Dopamine
ETA	Gq	Endothelin
ETB	Gq	Endothelin
FPR1	N/I	Formylpeptide
FPRL1	N/I	formylpeptide
GALR1	Gi	Galanin
GALR2	Gi/Gq	Galanin
GIP	N/I	Gastric inhibitory polypeptide
mGluR1	Gq	Glutamate
GPR5	N/I	Single C motif-1 (SMC-1)
GPR24 (also known as MCH or SLC-1)	Gi/Gq	Melanin Concentrating Hormone
GRPR	Gq	Gastrin releasing peptide
M1	Gq	Acetylcholine
M2	Gi	Acetylcholine
M3	Gq	Acetylcholine
M4	Gi	Acetylcholine

M5	Gq	Acetylcholine
MC3	Gs	Melanocortin
NK1R	Gq	Substance P
NK2R	Gq	Neurokinin-A
NK3R	Gq	Neurokinin-B
NMBR	Gq	Neuromedin B
NPY5	Gi	Neuropeptide Y
NTSR1	Gq	Neurotensin
NTSR2	Gq	Neurotensin
OPRD	Gi	Opiod
OPRL1	Gi	Opiod
OPRK	Gi	Opiod
OPRM	Gi	Opiod
OPRM1A	Gi	Opiod
OX_1R	N/I	Orexin
OX_2R	N/I	Orexin
PACAP	Gs	Pituitary adenylyl cyclase activating peptide
PAF	N/I	Platelet activating factor
PGE EP1	N/I	Prostaglandin
PGE EP2	Gs	Prostaglandin
PGE EP4	N/I	Prostaglandin
PTHR1	N/I	Parathyroid hormone
PTHR2	N/I	Parathyroid hormone
SCTR	Gs	Secretin
SST1	Gi	Somatostatin
SST2	Gi	Somatostatin
SST3	Gi	Somatostatin
SST4	Gi	Somatostatin
SST5	Gi	Somatostatin
TSHR	Gs	Thyroid Stimulating Hormone
VIPR	Gs	Vasoactive Intestinal Peptide
VIPR2	Gs	Vasoactive Intestinal Peptide

Note: N/I means not indicated

B. Receptor Screening

Screening candidate compounds against a non-endogenous, constitutively activated version of the known GPCRs disclosed herein allows for the direct identification of candidate compounds which act at the cell surface of the receptor, without requiring use of the receptor's endogenous ligand. By determining areas within the body where the endogenous version of known GPCRs disclosed herein is expressed and/or over-expressed, it is possible to determine related disease/disorder states which are associated with the

expression and/or over-expression of the receptor; such an approach is disclosed in this patent document.

Table C below lists the known GPCRs and tissues within the body are expressed and/or over-expressed. The listed references provide support for such tissue expression.

TABLE C

Known GPCRs	Location of Expression	Reference
5HT-1A	N/I	N/I
5HT-1B	Striatum	Jin, H. et al., 267(9) J Biol Chem 5735 (1992)
5HT-1D	Cerebral cortex	Weinshank, R.L. et al., 89(8) Proc Natl Acad Sci U S A 3630 (1992)
5HT-1E	N/I	N/I
5HT-1F	Brain	Adham, N. et al., 90 Proc Natl Acad Sci USA 408 (1993)
5HT-2B	Various tissues, including Brain	Kursar, J.D., 46(2_ Mol Pharmacol 227 (1994)
5HT-4A	Brain, Intestine and Atrium	Blondel, O. et al., 70 J Nerochem 2252 (1998)
5HT-4B	Brain, Intestine and Atrium	Blondel, O. et al., 70 J Nerochem 2252 (1998)
5HT-4C	Brain, Intestine and Atrium	Blondel, O. et al., 70 J Nerochem 2252 (1998)
5HT-4D	Intestine	Blondel, O. et al., 70 J Neurochem 2252 (1998)
5HT-4E	Brain	Claeysen, S. et al., 55(5) Mol Pharmacol 910 (1999)
5HT-5A	Brain	Rees, S. et al., 355(3) FEBS Lett 242 (1994)
5HT-6	Caudate Nucleus	Kohen, R. et al., 66(1) J Neurochem 47 (1996)
5HT-7	Brain, Coronary artery	Bard, J.A. et al., 268(31) J Biol Chem 23422 (1993)
AVPR1A	N/I	N/I
AVPR1B	Pituitary	Sugimoto, T. et al., 269(43) J. Biol. Chem 27088 (1994)
AVPR2	Lung, Kidney	Fay, M.J., et al., 17(3) Peptides 477 (1996)
BBR3	Testis, Lung carcinoma	Fathi, Z. et al., 268(8) J. Biol. Chem. 5979 (1993)
BDKR1	N/I	N/I
BDKR2	N/I	N/I
C3a	Lung, Spleen, Ovary, Placenta, Small Intestine and Brain	Ames, R. et al., 271(34) J. Biol. Chem 20231 (1996)
C5a	N/I	N/I
CB1	Brain	Gerard, C.M. et al., 279

		Biochem J. 129 (1991)
CB2	Spleen, Macrophage	Munro, S. et al., 365(6441) Nature 61 (1993)
CCR2b	N/I	N/I
CCR3	Endometrium	Zhang, J. et al., 62(2) Biol Reprod 404 (2000)
CCR5	Thymus, Spleen	Raport, C.J. et al., 271(29) J Biol Chem 17161 (1996)
CCR8	Thymus, Spleen and Lymph nodes	Napolitano M. et al., Forum (Geneva) 1999 Oct-Dec;9(4):315-24
CCR9	Thymus	Zaballos, A. et al., 162(10) J. Immunol 5671 (1999)
CRFR1	Brain, Pituitary	Perrin, M.H. et al., 133(6) Endocrinology 3058 (1993)
CXCR4	Colonic epithelial cells	Jordan, N.J. et al., 104(8) J Clin Invest 1061 (1999)
Dopamine D1	Caudate, Nucleus accumbens and Olfactory tubercle	Dearry, A. et al., 347 Nature 72 (1990)
Dopamine D2	Retina	Dearry, A. et al., 11(5) Cell Mol. Neurobiol. 437 (1991)
Dopamine D3	Brain	Schmauss, C. et al., 90(19) Proc Natl Acad Sci USA 8942 (1993)
Dopamine D5	Brain	Sunahara, R.K. et al., 350(6319) Nature 614 (1991)
ETA	Placenta, Uterus, Testis, Adrenal gland	Adachi, M. et al., 180(3) Biochem Biophys Res Commun 1265 (1991)
ETB	N/I	N/I
FPR1	N/I	N/I
FPRL1	N/I	N/I
GALR1	Hypothalamic paraventricular, Supraoptic nuclei	Gundlach, A.L. et al., 863 Ann NY Acad Sci 241 (1998)
GALR2	Hypothalamus, Hippocampus, Anterior pituitary	Fathi, Z. et al., 51 Brain Res Mol Brain Res 49 (1997)
GIP	N/I	N/I
mGluR1	Brain	Stephan, D. et al., 35(12) Neuropharmacology 1649 (1996)
GPR5	Leukocyte cells	Shan, L. et al., 268(3) Biochem Biophys Res Commun 938 (2000)
GPR24 (also known as MCH or SLC-1)	Fore-brain, Hypothalamus	Kolakowki LF Jr. et al., 398(2-3) FEBS Lett 253 (1996)
GRPR	Lung carcinoma cells	Corjay, M.H. et al., 266 Jo Biol Chem 18771 (1991)
M1	Heart, Pancreas and Neuronal cell lines	Peralta, E.G. et al., Embo J. 6(13) 3923 (1987)
M2	Heart, Pancreas and Neuronal cell lines	Peralta, E.G. et al., Embo J. 6(13) 3923 (1987)
M3	Heart, Pancreas and Neuronal cell lines	Peralta, E.G. et al., Embo J. 6(13) 3923 (1987)

M4	Heart, Pancreas and Neuronal cell lines	Peralta, E.G. et al., Embo J. 6(13) 3923 (1987)
M5	Brain	Bonner, T.I. et al., Neuron 1(5), 403 (1988)
MC3	Brain, Placenta, Gut	Gantz I. et al., 268(11) Jo Biol Chem 8246 (1993)
NK1R	Spinal cord, Lung	Taked, Y. et al., 179(3) Biochem Biophys Res Commun 1232 (1991)
NK2R	N/I	N/I
NK3R	N/I	N/I
NMBR	Lung carcinoma cells	Corjay,M.H. et al., 266 Jo Biol Chem 18771 (1991)
NPY5	Hypothalamus	Gerald, C. et al., 382 Nature 168 (1996)
NTSR1	Brain, Small intestine	Vita, N. et al., 17 (1-2) FEBS Lett 139 (1993)
NTSR2	N/I	N/I
OPRD	Peripheral blood lymphocytes	Wick, M.J. et al., 64(1) J. Neuroimmunol 29 (1996)
OPRL1	N/I	N/I
OPRK	Placenta, Brain	Manson, E. et al., 202(3) Biochem Biophys Res Commun 1431 (1994)
OPRM	N/I	N/I
OPRM1A	N/I	N/I
OX_1R	Hypothalamus	Sakurai T. et al., 92 Cell 573 (1998)
OX_2R	Hypothalamus	Sakurai T. et al., 92 Cell 573 (1998)
PACAP	Brain	Ogi, K. et al., 196(3) Biochem Biophys Res Commun 1511 (1993)
PAF	N/I	N/I
PGE EP1	Kidney	Watabe, A. et al., 268(27) J Biol Chem 20175 (1993)
PGE EP2	Small Intestine	Bastien, L. et al., 269(16) J. Biol. Chem 11873 (1994)
PGE EP4	N/I	N/I
PTHR1	Bone, Kidney	Schipani, E. et al., 132(5) Endocrinology 2157 (1993)
PTHR2	Brain, Pancreas	Usdin, T.B. et al., 270(26) J. Biol Chem 15455 (1995)
SCTR	Pancreas, Intestine	Chow, B.K., 212(1) Biochem Biophys Res Commun 204 (1995)
SST1	Jejunum, Stomach	Yamada Y. et al., 89 Proc Natl Acad Sci USA 251 (1992)
SST2	Cerebrum, Kidney	Yamada Y. et al., 89 Proc Natl Acad Sci USA 251 (1992)
SST3	Brain, Pancreatic islet	Yamada Y. et al., 6 Mol Endocrinol 2136 (1992)
SST4	Fetal, Adult Brain, Lung	Rohser L. et al., 90(9) Pro Natl Acad Sci USA 4146

		(1993)
SST5	Pituitary	Panetta R. et al., 45(3) mol Pharmacol 417 (1994)
TSHR	Retro-orbital tissues, Exophthalmos	Feliciello A. et al 342 Lancet 337 (1993)
VIPR	Lung	Sreedharan, S.P. et al., 193(2) Biochem Biophys Res Commun 546 (1993)
VIPR2	Skeletal muscle	Adamou, J.E. et al., 209(2) Biochem Biophys Res Commun 385 (1995)

Note: N/I means not indicated

Creation of a non-endogenous version of a known GPCR that may evidence constitutive activity is most preferably based upon the distance from a proline residue located within TM6 of the GPCR; this technique is disclosed in co-pending and commonly assigned patent document U.S. Serial Number 09/170,496, incorporated herein by reference. This technique is not predicated upon traditional sequence "alignment" but rather a specified distance from the aforementioned TM6 proline residue. By mutating the amino acid residue located 16 amino acid residues from this residue (presumably located in the IC3 region of the receptor) to, most preferably, a lysine residue, such activation may be obtained. Other amino acid residues may be used for the mutation, but lysine is most preferred.

D. Disease/Disorder Identification and/or Selection

As will be set forth in greater detail below, most preferably inverse agonists, partial agonists and agonists in the form of small molecule chemical compounds to the non-endogenous, constitutively activated GPCR can be identified by the methodologies of this invention. Such compounds are ideal candidates as lead modulators in drug discovery programs for treating diseases or disorders associated with a particular receptor. The ability

to directly identify such compounds to the GPCR, in the absence of use of the receptor's endogenous ligand, allows for the development of pharmaceutical compositions.

Preferably, in situations where it is unclear what disease or disorder may be associated with a receptor; the DNA sequence of the known GPCR is used to make a probe for (a) dot-blot analysis against tissue-mRNA, and/or (b) RT-PCR identification of the expression of the receptor in tissue samples. The presence of a receptor in a tissue source, or a diseased tissue, or the presence of the receptor at elevated concentrations in diseased tissue compared to a normal tissue, can be preferably utilized to identify a correlation with a treatment regimen, including but not limited to, a disease associated with that disease. Receptors can equally well be localized to regions of organs by this technique. Based on the known functions of the specific tissues to which the receptor is localized, the putative functional role of the receptor can be deduced.

E. Screening of Candidate compounds

1. Generic GPCR screening assay techniques

When a G protein receptor becomes constitutively active, it binds to a G protein (*e.g.*, Gq, Gs, Gi, Gz, Go) and stimulates the binding of GTP to the G protein. The G protein then acts as a GTPase and slowly hydrolyzes the GTP to GDP, whereby the receptor, under normal conditions, becomes deactivated. However, constitutively activated receptors continue to exchange GDP to GTP. A non-hydrolyzable analog of GTP, $[^{35}S]GTP\gamma S$, can be used to monitor enhanced binding to membranes which express constitutively activated receptors. It is reported that $[^{35}S]GTP\gamma S$ can be used to monitor G protein coupling to membranes in the absence and presence of ligand. An example of this monitoring, among other examples well-known and available to those in the art, was

reported by Traynor and Nahorski in 1995. The preferred use of this assay system is for initial screening of candidate compounds because the system is generically applicable to all G protein-coupled receptors regardless of the particular G protein that interacts with the intracellular domain of the receptor.

2. Specific GPCR screening assay techniques

Once candidate compounds are identified using the "generic" G protein-coupled receptor assay (*i.e.*, an assay to select compounds that are agonists, partial agonists, or inverse agonists), further screening to confirm that the compounds have interacted at the receptor site is preferred. For example, a compound identified by the "generic" assay may not bind to the receptor, but may instead merely "uncouple" the G protein from the intracellular domain.

a. Gs, Gz and Gi.

Gs stimulates the enzyme adenylyl cyclase. Gi (and Gz and Go), on the other hand, inhibit this enzyme. Adenylyl cyclase catalyzes the conversion of ATP to cAMP; thus, constitutively activated GPCRs that couple the Gs protein are associated with increased cellular levels of cAMP. On the other hand, constitutively activated GPCRs that couple Gi (or Gz, Go) protein are associated with decreased cellular levels of cAMP. *See, generally*, "Indirect Mechanisms of Synaptic Transmission," Chpt. 8, From Neuron To Brain (3rd Ed.) Nichols, J.G. et al eds. Sinauer Associates, Inc. (1992). Thus, assays that detect cAMP can be utilized to determine if a candidate compound is, *e.g.*, an inverse agonist to the receptor (*i.e.*, such a compound would decrease the levels of cAMP). A variety of approaches known in the art for measuring cAMP can be utilized; a most preferred approach relies upon the use of anti-cAMP antibodies in an ELISA-based format. Another type of assay that can be utilized is a second messenger reporter system assay. Promoters on genes drive

the expression of the proteins that a particular gene encodes. Cyclic AMP drives gene expression by promoting the binding of a cAMP-responsive DNA binding protein or transcription factor (CREB) that then binds to the promoter at specific sites called cAMP response elements and drives the expression of the gene. Reporter systems can be constructed which have a promoter containing multiple cAMP response elements before the reporter gene, *e.g.*, β-galactosidase or luciferase. Thus, a constitutively activated Gs-linked receptor causes the accumulation of cAMP that then activates the gene and expression of the reporter protein. The reporter protein such as β-galactosidase or luciferase can then be detected using standard biochemical assays (Chen et al. 1995).

b. Go and Gq.

Gq and Go are associated with activation of the enzyme phospholipase C, which in turn hydrolyzes the phospholipid PIP_2, releasing two intracellular messengers: diacycloglycerol (DAG) and inistol 1,4,5-triphoisphate (IP_3). Increased accumulation of IP_3 is associated with activation of Gq- and Go-associated receptors. *See, generally,* "Indirect Mechanisms of Synaptic Transmission," Chpt. 8, From Neuron To Brain (3^{rd} Ed.) Nichols, J.G. et al eds. Sinauer Associates, Inc. (1992). Assays that detect IP_3 accumulation can be utilized to determine if an candidate compound is, *e.g.*, an inverse agonist to a Gq- or Go-associated receptor (*i.e.*, such a compound would decrease the levels of IP_3). Gq-associated receptors can also be examined using an AP1 reporter assay in that Gq-dependent phospholipase C causes activation of genes containing AP1 elements; thus, activated Gq-associated receptors will evidence an increase in the expression of such genes, whereby inverse agonists thereto will evidence a decrease in such expression, and agonists will evidence an increase in such expression. Commercially available assays for such detection are available.

3. Ligand-Based Confirmation Assays

The candidate compounds directly identified using the techniques (or equivalent techniques) above are then, most preferably, verified using a ligand-based verification assay, such as the one set forth in the protocol of Example 8. The importance here is that the candidate compound be directly identified; subsequent confirmation, if any, using the endogenous ligand, is merely to confirm that the directly identified candidate compound has targeted the receptor.

For example, sumatriptan is a well-known agonist of the 5-HT1B and 5-HT1D receptors, while naltrindole is a well-known antagonist to the OPM1D receptor. Accordingly, an agonist (sumatriptan) and/or antagonist (naltrindole) competitive binding assay(s) can be used to confirm that those candidate compounds directly identified using a ligand independent screening technique comprising non-endogenous, constitutively activated 5-HT1B or 5-HT1D, and non-endogenous constitutively activated OPM1D, respectfully, may be used for confirmatory purposes. Those skilled in the art are credited with the ability to select techniques for ligand-based confirmation assays.

4. GPCR Fusion Protein

The use of a non-endogenous, constitutively activated GPCR, for use in screening of candidate compounds for the direct identification of inverse agonists, agonists and partial agonists, provides an interesting screening challenge in that, by definition, the receptor is active even in the absence of an endogenous ligand bound thereto. Thus, in order to differentiate between, *e.g.,* the non-endogenous receptor in the presence of a candidate compound and the non-endogenous receptor in the absence of that compound, with an aim of such a differentiation to allow for an understanding as to whether such compound may be an inverse agonist, agonist, partial agonist or has no affect on such a receptor, it is preferred

that an approach be utilized that can enhance such differentiation. A preferred approach is the use of a GPCR Fusion Protein.

Generally, once it is determined that a non-endogenous GPCR has been constitutively activated using the assay techniques set forth above (as well as others), it is possible to determine the predominant G protein that couples with the endogenous GPCR. Coupling of the G protein to the GPCR provides a signaling pathway that can be assessed. Because it is most preferred that screening take place by use of a mammalian expression system, such a system will be expected to have endogenous G protein therein. Thus, by definition, in such a system, the non-endogenous, constitutively activated GPCR will continuously signal. In this regard, it is preferred that this signal be enhanced such that in the presence of, *e.g.*, an inverse agonist to the receptor, it is more likely that it will be able to more readily differentiate, particularly in the context of screening, between the receptor when it is contacted with the inverse agonist.

The GPCR Fusion Protein is intended to enhance the efficacy of G protein coupling with the non-endogenous GPCR. The GPCR Fusion Protein is preferred for screening with a non-endogenous, constitutively activated GPCR because such an approach increases the signal that is most preferably utilized in such screening techniques. This is important in facilitating a significant "signal to noise" ratio; such a significant ratio is preferred for the screening of candidate compounds as disclosed herein.

The construction of a construct useful for expression of a GPCR Fusion Protein is within the purview of those having ordinary skill in the art. Commercially available expression vectors and systems offer a variety of approaches that can fit the particular needs of an investigator. The criteria of importance for such a GPCR Fusion Protein construct is that the endogenous GPCR sequence and the G protein sequence both be in-frame

(preferably, the sequence for the endogenous GPCR is upstream of the G protein sequence) and that the "stop" codon of the GPCR must be deleted or replaced such that upon expression of the GPCR, the G protein can also be expressed. The GPCR can be linked directly to the G protein, or there can be spacer residues between the two (preferably, no more than about 12, although this number can be readily ascertained by one of ordinary skill in the art). Use of a spacer is preferred (based upon convenience) in that some restriction sites that are not used will, effectively, upon expression, become a spacer. Most preferably, the G protein that couples to the non-endogenous GPCR will have been identified prior to the creation of the GPCR Fusion Protein construct. Because there are only a few G proteins that have been identified, it is preferred that a construct comprising the sequence of the G protein (*i.e.,* a universal G protein construct) be available for insertion of an endogenous GPCR sequence therein; this provides for efficiency in the context of large-scale screening of a variety of different endogenous GPCRs having different sequences.

F. Co-transfection of a Target Gi Coupled GPCR with a Signal-Enhancer Gs Coupled GPCR (cAMP Based Assays)

A Gi coupled receptor is known to inhibit adenylyl cyclase, and, therefore, decrease the level of cAMP production, which can make assessment of cAMP levels challenging. An effective technique in measuring the decrease in production of cAMP as an indication of constitutive activation of a receptor that predominantly couples Gi upon activation can be accomplished by co-transfecting a signal enhancer, *e.g.,* a non-endogenous, constitutively activated receptor that predominantly couples with Gs upon activation (*e.g.*, TSHR-A623I, disclosed below), with the Gi linked GPCR (such a technique is exemplified herein with the Gi coupled receptor, GPR24). As is apparent, constitutive activation of a Gs coupled

receptor can be determined based upon an increase in production of cAMP. Constitutive activation of a Gi coupled receptor leads to a decrease in production cAMP. Thus, the co-transfection approach is intended to advantageously exploit these "opposite" affects. For example, co-transfection of a non-endogenous, constitutively activated Gs coupled receptor (the "signal enhancer") with the endogenous Gi coupled receptor (the "target receptor") provides a baseline cAMP signal (*i.e.*, although the Gi coupled receptor will decrease cAMP levels, this "decrease" will be relative to the substantial increase in cAMP levels established by constitutively activated Gs coupled signal enhancer). By then co-transfecting the signal enhancer with a constitutively activated version of the target receptor, cAMP would be expected to further decrease (relative to base line) due to the increased functional activity of the Gi target (*i.e.*, which decreases cAMP).

Screening of candidate compounds using a cAMP based assay can then be accomplished, with two provisos: first, relative to the Gi coupled target receptor, "opposite" effects will result, *i.e.*, an inverse agonist of the Gi coupled target receptor will increase the measured cAMP signal, while an agonist of the Gi coupled target receptor will decrease this signal; second, as would be apparent, candidate compounds that are directly identified using this approach should be assessed independently to ensure that these do not target the signal enhancing receptor (this can be done prior to or after screening against the co-transfected receptors).

G. Medicinal Chemistry

Generally, but not always, direct identification of candidate compounds is preferably conducted in conjunction with compounds generated via combinatorial chemistry techniques, whereby thousands of compounds are randomly prepared for such analysis. Generally, the results of such screening will be compounds having unique core structures;

thereafter, these compounds are preferably subjected to additional chemical modification around a preferred core structure(s) to further enhance the medicinal properties thereof. Such techniques are known to those in the art and will not be addressed in detail in this patent document.

H. Pharmaceutical compositions

Candidate compounds selected for further development can be formulated into pharmaceutical compositions using techniques well known to those in the art. Suitable pharmaceutically-acceptable carriers are available to those in the art; for example, see Remington's Pharmaceutical Sciences, 16th Edition, 1980, Mack Publishing Co., (Oslo et al., eds.).

I. Other Utility

Although a preferred use of the non-endogenous version of the known GPCRs disclosed herein may be for the direct identification of candidate compounds as inverse agonists, agonists or partial agonists (preferably for use as pharmaceutical agents), these versions of known GPCRs can also be utilized in research settings. For example, *in vitro* and *in vivo* systems incorporating GPCRs can be utilized to further elucidate and better understand the roles these receptors play in the human condition, both normal and diseased, as well as understanding the role of constitutive activation as it applies to understanding the signaling cascade. The value in non-endogenous known GPCRs is that their utility as a research tool is enhanced in that, because of their unique features, non-endogenous known GPCRs can be used to understand the role of these receptors in the human body before the endogenous ligand therefor is identified. Other uses of the disclosed receptors will become apparent to those in the art based upon, *inter alia*, a review of this patent document.

EXAMPLES

The following examples are presented for purposes of elucidation, and not limitation, of the present invention. While specific nucleic acid and amino acid sequences are disclosed herein, those of ordinary skill in the art are credited with the ability to make minor modifications to these sequences while achieving the same or substantially similar results as reported below. The traditional approach to application or understanding of sequence cassettes from one sequence to another (*e.g.* from rat receptor to human receptor or from human receptor A to human receptor B) is generally predicated upon sequence alignment techniques whereby the sequences are aligned in an effort to determine areas of commonality. The mutational approaches disclosed herein do not rely upon a sequence alignment approach but are instead based upon an algorithmic approach and a positional distance from a conserved proline residue located within the TM6 region of GPCRs. Once this approach is secured, those in the art are credited with the ability to make minor modifications thereto to achieve substantially the same results (*i.e.*, constitutive activation) disclosed herein. Such modified approaches are considered within the purview of this disclosure

Example 1
PREPARATION OF ENDOGENOUS KNOWN GPCRS

A. Expression By Standard PCR

PCR was performed using a specific cDNA as template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides.

The resulting PCR fragment was digested with the respective restriction sites and cloned into a pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified. See Table D below:

TABLE D

Receptor Identifier	Template	Cycle Conditions Min ('), Sec (")	5' Primer (SEQ.ID.NO.) and Restriction site	3' Primer (SEQ.ID.NO.) and Restriction site
5HT-1A	Genomic DNA	94° for 1' 60°C for 1' 72° for 1' 30"	CGGAAGCTTAGC CATGGATGTGCT CAGCCCTGGTCA (1); HindIII	CCGGAATTCCTG GCGGCAGAAGTT ACACTTAATG (2); EcoRI
5HT-1B	Genomic DNA	94° for 1' 60°C for 1' 72° for 1' 30"	TCCAAGCTTGGG GCGAGGAGAGCC ATGGAGGA (3); HindIII	GGCGAATTCACTT GTGCACTTAAAA CGTATCAGTT (4); EcoRI
5HT-1D	Genomic DNA	94° for 1' 60°C for 1' 72° for 1' 30"	ATCTACCATGTC CCCACTGAACCA GTCAGC (5)	ATAGAATTCGGA GGCCTTCCGGAA AGGGACAA (6); EcoRI
5HT-1E	Genomic DNA	94° for 1' 60°C for 1' 72° for 2' 10"	CCACAGTGTCGA CTGAAACAAGGG AAACATGAAC (7); SalI	CAGTATGCTCTCG GCATCTAATGAG (8)
5HT-1F	Genomic DNA	94° for 1' 60°C for 1' 72° for 2' 10"	ATCACCATGGAT TTCTTAAATTCAT CTGATC (9)	TTAGGATCCACAT CGACATCGCACA AGCTTTTG (10); BamHI
5HT-2B	Uterus cDNA	94° for 1' 60°C for 1' 72° for 1' 30"	GAAAAGCTTGCC ATGGCTCTCTCTT ACAGAGTGTCTG (11); HindIII	GTTGGATCCTACA TAACTAACTTGCT CTTCAGTTT (12); BamHI
5HT-4A	Brain cDNA	94° for 1' 60°C for 1' 72° for 1' 30"	ATCACCATGGAC AAACTTGATGCT AATGTGAG (13)	CCTGAATTCGAA GCATGATTCCAG GGATTCTGG (14); EcoRI
5HT-4B	Brain cDNA	94° for 1' 60°C for 1' 72° for 1' 30"	ATCACCATGGAC AAACTTGATGCT AATGTGAG (15)	AGGGAATTCAGT GTCACTGGGCTG AGCAGCCAC (16); EcoRI
5HT-4C	Brain cDNA	94° for 1' 60°C for 1' 72° for 1' 30"	ATCACCATGGAC AAACTTGATGCT AATGTGAG (17)	TTGGAATTCGGAT GGTTTGGTCAATC TTCTCTTC (18); EcoRI
5HT-4D	5HT-4E DNA	94° for 1' 60°C for 1' 72° for 2"	ATCACCATGGAC AAACTTGATGCT AATGTGAG (19)	AGGGAATTCAAA TCTTAGTACATGT GTGGATCCATTA AT (20); EcoRI

5HT-4E	Brain cDNA	94° for 1' 60°C for 1' 72° for 1' 15"	ATCACCATGGAC AAACTTGATGCT AATGTGAG (21)	TCAGAATTCGAC AGGAACTGGTCT ATTGCAGAA (22); EcoRI
5HT-5A	Brain cDNA	94° for 1' 60°C for 1' 72° for 2' 10"	CCTAAGCTTGCC ATGGATTTACCA GTGAACCTAACC TCC (23); HindIII	TCTGAATTCGTGT TGCCTAGAAAAG AAGTTCTTGA (24); EcoRI
5HT-6	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
5HT-7	Brain cDNA	94°C for 1' 72°C for 2"	AGCGGAATTCGG CGGCGCGATGAT GGACGTT (25); EcoRI	TTTCGGATCCATT GTTCTGCTTTCAA TCAT (26); BamHI
AVPR1A	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
AVPR1A variant	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
AVPR1B	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
AVPR2	IMAGE 301449	pfu PCR 94° for 1' 63°C for 1' 72° for 2"	CAGGAATTCAGA ACACCTGCCCCA GCCCCAC (27); EcoRI	AGCGGATCCCGA TGAAGTGTCCTTG GCCAGGGA (28); BamHI
BBR3	Uterus cDNA	94°C for 1' 56°C for 1' 72°C for 1'20"	ACAGAATTCAGA AGAAATGGCTCA AAGGCA (29); EcoRI	CATGGATCCTTGA AAAGCTAGAAAC TGTCC (30); BamHI
BDKR1	IMAGE 1472696	pfu PCR 94° for 1' 65°C for 1' 72° for 2"	TGTAAGCTTCAG GTCACTGTGCAT GGCATCATC (31); HindIII	GCTGGATCCATTC CGCCAGAAAAGT TGGAAGATTTC (32); BamHI
BDKR2	IMAGE 1682455	pfu PCR 94° for 1' 65°C for 1' 72° for 2"	ACTAAGCTTCCA AATGTTCTCTCCC TGGAAGATA (33) HindIII	GTTGAATTCCTGT CTGCTCCCTGCCC AGTCCTG (34); EcoRI
C3a	Genomic DNA	94° for 1' 65°C for 1' 72° for 1' 30"	CAGAAGCTTAGC AATGGCGTCTTT CTCTGCTG (35); HindIII	ACAGGATCCCAC AGTTGTACTATTT CTTTCTGAAATG (36); BamHI
C5a	Thymus	94° for 1' 65°C for 1' 72° for 1' 10"	GGGAAGCTTAGG AGACCAGAACAT GAACTCCTTC (37); HindIII	TGTGAATTCCACT GCCTGGGTCTTCT GGGCCAT (38); EcoRI
CB1	EST 01536	pfu PCR 94° for 1' 65°C for 1' 72° for 2' 30"	GGGAAGCTTTCT CAGTCATTTTGA GCTCAGCC (39); HindIII	TCAGAATTCCAG AGCCTCGGCAGA CGTGTCTGT (40); EcoRI
CB2	IMAGE 1301708	pfu PCR 94° for 1' 60°C for 1' 72° for 2"	CAAAAGCTTCTA GACAAGCTCAGT GGAATCTGA (41); HindIII	GCCGAATTCGCA ATCAGAGAGGTC TAGATCTCTG (42); EcoR1
CCR2b	Genomic DNA	94° for 1' 60°C for 1'	GACAAGCTTCCC CAGTACATCCAC	CTCGGATCCTAA ACCAGCCGAGAC

		72° for 1'10"	AACATGC (43); HindIII	TTCCTGCTC (44); BamHI
CCR3	Genomic DNA	94° for 1' 60°C for 1' 72° for 1'10"	ATCGCCATGACA ACCTCACTAGAT ACAGTTGAG (45)	TCTGAATTCAAAC ACAATAGAGAGT TCCGGCTC (46); EcoRI
CCR5	Genomic DNA	94° for 1' 62°C for 1' 72° for 1'10"	GCAAAGCTTGGA ACAAGATGGATT ATCAAGTGTC (47); HindIII	TCCGGATCCCAA GCCCACAGATAT TTCCTGCTC (48); BamHI
CCR8	Genomic DNA	94° for 1' 60°C for 1' 72° for 1'10"	TGAAAGCTTCCC GCTGCCTTGATG GATTATAC (49); HindIII	TGAGAATTCCAA AATGTAGTCTAC GCTGGAGGAA (50); EcoRI
CCR9	Genomic DNA	94° for 1' 60°C for 1' 72° for 1'10"	ATCACCATGACA CCCACAGACTTC ACAAGCCCTATT CCTAACATGGCT GATGACTATGG (51)	GACGAATTCGAG GGAGAGTGCTCC TGAGGTTGT (52); EcoRI
CRFR1	Pituitary cDNA	94° for 1' 65°C for 1' 72° for 1'20"	ATCACCATGGGA GGGCACCCGCAG CTCCGT (53)	CGGGAATTCGAC TGCTGTGGACTGC TTGATGCT (54); EcoRI
CXCR4	Genomic DNA	94° for 1' 65°C 72° for 1'	ATCACCATGGAG GGGATCAGTATA TACACTTCAGAT AACTACACCGAG GAAATG (55)	TCTGAATTCGCTG GAGTGAAAACTT GAAGACTCAG (56); EcoRI
Dopamine D1	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
Dopamine D2	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
Dopamine D3	Brain cDNA	94°C for 1' 62° for 1'20" 72°C for 1'20"	AAGAAGCTTGGC ATCACGCACCTC CTCTGG (57); HindIII	GGCTCTAGAAAT GGGTACAAAGAG TGTT (58); XbaI
Dopamine D5	Genomic DNA	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
ETA	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
ETB	IMAGE 1086987	pfu PCR 94° for 1' 60°C for 1' 72° for 2' 20"	CGGAAGCTTCTG GAGCAGGTAGCA GCATG (59); HindIII	CTTGGATCCAGAT GAGCTGTATTTAT TACTGGAACG (60); BamHI
FPR1	IMAGE 2153284	94°C for 1' 63° for 1' 72°C for 2'30"	ATCACCATGGAG ACAAATTCCTCT CTCCCC (61)	CCCGAATTCCTTT GCCTGTAACTCCA CCTCTGC (62); EcoRI
FPRL1	Genomic DNA	94°C for 1' 65° for 1' 72°C for 1'10"	GCAAAGCTTGCT GCTGGCAAGATG GAAACCAAC (63); HindIII	CCAGAATTCCATT GCCTGTAACTCA GTCTCTGC (64); EcoRI
GALR1	Stomach cDNA	94°C for 1' 60° for 1'20" 72°C for 1'20"	CCGGAATTCGCC GGGACAGCCCCG CGGGCC (65);	GCAGGATCCTTAT CACACATGAGTA CAATTGGT (66);

			EcoRI	BamHI
GALR2	Hippocampus cDNA	94°C for 1' 62° for 1'20" 72°C for 1'20"	GGCGAATTCGGG GTCAGCGGCACC ATGAACG (67); EcoRI	GTGGGATCCCAG CGCGCCCGCTAA GTGCT (68); BamHI
GIP	Brain cDNA	94° for 1' 65°C for 1' 72° for 1'30"	CAGAAGCTTCGC CGCCCTCACGAT GACTAC (69); HindIII	CGCGAATTCGCA GTAACTTTCCAAC TCCCGGCT (70); EcoRI
mGluR1	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
GPR5	Genomic DNA	94°C for 1' 64° for 1' 72°C for 1'30"	TATGAATTCAGA TGCTCTAAACGT CCCTGC (71); EcoRI	TCCGGATCCACCT GCACCTGCGCCT GCACC (72); BamHI
GPR24 (also known as MCH or SLC-1)	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
GRPR	Stomach cDNA	94°C for 1' 56° for 1'20" 65°C for 1'20"	AGGAAGCTTTTA GGTGGGAAAAA AAATCTA (73); HindIII	CCGGAATTCAAG GGGCAAAATCAA GGGTCAA (74); EcoRI
M1	Genomic DNA	94°C for 1' 60° for 1' 72°C for 1'50"	GCCAAGCTTAGC CACCATGAACAC TTCAGCCC (75); HindIII	GGAGAATTCGCA TTGGCGGGAGGG AGTGCGGTG (76); EcoRI
M2	Genomic DNA	94°C for 1' 60° for 1' 72°C for 1'50"	ATCACCATGAAT AACTCAACAAAC TCCTCTAAC (77)	GATGAATTCCCTT GTAGCGCCTATGT TCTTATA (78); EcoRI
M3	Genomic DNA	94°C for 1' 60° for 1' 72°C for 1'50"	ATCACCATGACC TTGCACAATAAC AGTACAAC (79)	CTCGAAATTCCA AGGCCTGCTCGG GTGCGCGCT (80); EcoRI
M4	Genomic DNA	94°C for 1' 60° for 1' 72°C for 1'50"	ATCACCATGGCC AACTTCACACCT GTCAA (81)	GCCGAATTCCCTG GCAGTGCCGATG TTCCGATA (82); EcoRI
M5	Genomic DNA	94°C for 1' 60° for 1' 72°C for 1'50"	ATCACCATGGAA GGGGATTCTTAC CACAAT (83)	GACGGATCCGGG TAGCTTGCTGTTC CCCTGCCA (84); BamHI
MC3	Genomic DNA	94°C for 1' 54° for 1'30" 72°C for 1'20"	CAGGAATTCTGA CAGCAATGAATG CTTCGT (85); EcoRI	AATGGATCCTATC CCAAGTTCATGCC GTTGCAG (86); BamHI
NK1R	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'50"	AGTAAGCTTTAC GCCTAGCTTCGA AATGGAT (87); HindIII	TGTGAATTCGGA GAGCACATTGGA GGAGAAGCT (88); EcoRI
NK2R	Uterus cDNA	94°C for 1' 65° for 1' 72°C for 1'50"	TCCAAGCTTAGA AGCAGCCATGGG GACCTGTGACA (89); HindIII	AACGAATTCAAT TTCAACATGAGTT TTGGTGGGGG (90); EcoRI
NK3R	Brain cDNA	94°C for 1'	ATCTGCAGACCG	ATGGGATCCAGA

		65° for 1'20" 72°C for 1'20"	GTGGCGATGGCC ACT (91)	ATATTCATCCACA GAGGTATAGG (92); BamHI
NMBR	Brain cDNA	94°C for 1' 65° for 1'20" 72°C for 1'20"	TGAGAATTCCAG CGGACTCTGCTG GAAAGGA (93); EcoRI	GTTGGATCCAGG TAGTGAGTTGAA TGGCCA (94); BamHI
NPY5	Genomic DNA	94°C for 1' 54° for 1'30" 72°C for 1'20"	GGAAAGCTTCAA GAAAGACTATAA TATGGAT (95); HindIII	GGAGGATCCAGT GAGAATTATTAC ATATGAAG (96); BamHI
NTSR1	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
NTSR2	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
OPRD	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'15	CGGAAGCTTGCA GCCATGGAACCG GCCCCCTCC (97); HindIII	GCCGAATTCGGC GGCAGCGCCACC GCCGGGACC (98); EcoRI
OPRL1	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'15"	AGTAAGCTTGCA GGGCAGTGGCAT GGAGCCC (99); HindIII	GCCGAATTCTGC GGGCCGCGGTAC CGTCTCAGA (100); EcoRI
OPRK	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'15"	TTTAAGCTTGCA GCACTCACCATG GAATCCCCGAT (101); HindIII	CTACTGGTTTATT CATCCCATCGATG TC (102);
OPRM	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
OPRM1A	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
OX_1R	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
OX_2R	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'20"	ACCAAGCTTGAG CCCGTGATGTCC GGCACC (103); HindIII	CAGGGATCCTTGT CATATGAATAAA TATT (104); BamHI
PACAP	Fetal Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'30"	AGTAAGCTTGGC CAAGAAGTGTCA TGGCTGGTG (105); HindIII	CATGAATTCGGT GGCCAGATTGTC AGCAGGGAG (106); EcoRI
PAF	Genomic DNA	94°C for 1' 63° for 1' 72°C for 2'30"	CTGAAGCTTCCA GCCCACAGCAAT GGAGCCA (107); HindIII	CAGGAATTCATTT TTGAGGGAATTG CCAGGGATCTG (108); EcoRI
PGE EP1	cDNA clone	pfu PCR 94°C for 1' 63° for 1' 72°C for 2'30"	ATCGCCATGAGC CCTTGCGGGCCC CTCAA (109)	TTGGAATTCGAA GTGGCTGAGGCC GCTGTGCCG (110); EcoRI
PGE EP2	Thymus cDNA	94°C for 1' 63° for 1' 72°C for 2'30"	GCAAAGCTTTTC CAGGCACCCCAC CATGGGC (111); HindIII	CTGGAATTCAAG GTCAGCCTGTTTA CTGGCATC (112); EcoRI

PGE EP4	cDNA clone	pfu PCR 94°C for 1' 60° for 1' 72°C for 2'30"	ATCATCATGTCC ACTCCCGGGGTC AAT (113)	TGCGAATTCTATA CATTTTTCTGATA AGTTCAGTGTT (114); EcoRI
PTHR1	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
PTHR2	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'50"	CTGAAGCTTCCT ACAGCCGTTCCG GGCATG (115); HindIII	CGAGAACATCCT CAGTTTCTCCTTG G (116)
SCTR	Small Intestine	94°C for 1' 65° for 1' 72°C for 1'45"	GGGAAGCTTGCG GGCACCATGCGT CCCCACCT (117); HindIII	AGCGAATTCGAT GATGCTGGTCCTG CAGGTGCC (118); EcoRI
SST1	Genomic DNA	94°C for 1' 65° for 1'20" 72°C for 1'20"	GCCGAATTCAGC TGGGATGTTCCC CAATGGC (119); EcoRI	CAGGGATCCTGC GTGGCCCGGGCT CAGAGCG (120); BamHI
SST2	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*	*See alternative approach below*
SST3	Genomic DNA	94°C for 1' 65° for 1'20" 72°C for 1'20"	ACGGAATTCCCC TCAGCCATGGAC ATGCTTC (121); EcoRI	TGGGATCCCCAG GCCCCTACAGGT AGCTG (122); BamHI
SST4	Genomic DNA	94°C for 1' 65° for 1'20" 72°C for 1'20"	GCCGAATTCAGC TGCCCTGCGCCG GCACCCC (123); EcoRI	GAGGGATCCACG CAGGGTGGGTAG GGGAAGG (124); BamHI
SST5	Genomic DNA	94°C for 1' 65° for 1'20" 72°C for 1'20"	TCTAAGCTTGCA GAGCCTGACGCA CCCCAG (125); HindIII	CCTGAATTCCTGG GGGTGACACGGG GCCGCC (126); EcoRI
TSHR	Genomic DNA	94°C for 1' 65° for 1' 72°C for 2'30"	GGCGAATTCGGA GGATGGAGAAAT AGCCCC (127); EcoRI	GTAGGATCCCCT ACCATTGTGAGT AGTGTA (128); BamHI
VIPR1	Lung cDNA	94°C for 1' 65° for 1' 72°C for 1'30"	CCGAAGCTTCAG GGCAGACCATGC GCCCGCCA (129); HindIII	TGGGAATTCGAC CAGGGAGACTTC GGCTTGGAA (130); EcoRI
VIPR2	Brain cDNA	94°C for 1' 65° for 1' 72°C for 1'30"	GCTAAGCTTGCC ATGCGGACGCTG CTGCCTCCCGCG (131); HindIII	GTGGAATTCGAT GACCGAGGTCTC CGTTTGCAG (132); EcoRI

B. Expression by Alternative Approaches

1. AVPR1A

The endogenous human AVPR1A was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 64°C for 1min and 72 °C for 1 min and 30 sec. The 5' PCR fragment was obtained utilizing genomic DNA, as a template, and the following primer set:

5'-ATCACCATGCGTCTCTCCGCCGGTCCCGA-3' (SEQ.ID.NO.: 133) and

5'-TTGTTCACCTCGATCATGGAGAAGA-3'- (SEQ.ID.NO.:134).

The 3' PCR fragment was obtained by pfu polymerase (Stratagene) using IMAGE 1055179, as a template, and the following primer set:

5'-CGCAGTACTTCGTCTTCTCCATGA-3' (SEQ.ID.NO.:135) and

5'-CAAGAATTCAGTTGAAACAGGAATGAATTTGATGG-3' (SEQ.ID.NO.:136).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 60°C and 72°C for 1 min 30 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase and SEQ.ID.NO.:133 and SEQ.ID.NO.:136 as primers. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C and 72°C for 2 min 10 sec.

The resulting PCR fragment was digested with EcoRI restriction site and cloned into an EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

2. AVPR1A Variant

The endogenous human AVPR1A variant was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30

cycles of 94°C for 1 min, 64°C for 1min and 72 °C for 1 min and 30 sec. The 5' PCR fragment was obtained utilizing genomic DNA, as a template, and :

SEQ.ID.NO.:133 and SEQ.ID.NO.:134 as primers.

The 3' PCR fragment was obtained by pfu polymerase (Stratagene) using IMAGE 1542469, as a template, and SEQ.ID.NO.:136 and

5'-ACAGAATTCTCCAGTTCTCATTTTCTTATCCGTAC-3' (SEQ.ID.NO.:137).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min 30 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and SEQ.ID.NO.:133 and SEQ.ID.NO.:136 as primers. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min 10 sec.

The resulting PCR fragment was digested with EcoRI restriction site and cloned into an EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

3. AVPR1B

The endogenous human AVPR1B was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. Both rounds of PCR had the following cycle condition: 30 cycles of 94°C for 1 min, 65°C for 1min and 72 °C for 2 sec. The first round of PCR utilized a pituitary DNA, as a template, and a 5' PCR primer that contained a HindIII site with the following sequence:

5'-GCAAAGCTTGCTCATGGATTCTGGGCCTCT-3' (SEQ.ID.NO.:138)

The 3' PCR primer contained an EcoRI site with the following sequence:

5'-TCTGAATTCAAAGATGATGGTCTCAGCGGTGCC-3'- (SEQ.ID.NO.:139).

The second round of PCR utilized pituitary DNA as a template and a 5' PCR primer contained a HindIII site with the following sequence:

5'-GCAAAGCTTGCTCATGGATTCTGGGCCTCTGTGGG -3' (SEQ.ID.NO.:140)

and the 3' PCR primer contained an EcoRI site with the following sequence:

5'-TCTGAATTCAAAGATGATGGTCTCAGCGGTGCCTTCCC -3' (SEQ.ID.NO.:141).

The resulting PCR fragment was digested with HindIII and EcoRI restriction site and cloned into a HindIII-EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

4. 5HT6

The endogenous human 5HT6 receptor was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. Both rounds of PCR had the following cycle condition: 30 cycles of 94°C for 1 min, 60°C for 1min and 72 °C for 1 min and 45 sec. The first round of PCR utilized a caudate nucleus DNA, as a template, and a 5' PCR primer that contained a HindIII site with the following sequence:

5'-CATAAGCTTTCCCGCCACCCTATCACT-3' (SEQ.ID.NO.:142)

The 3' PCR primer contained an EcoRI site with the following sequence:

5'-ACTGAATTCTGCTCAATCCAGCTCCCCA-3'- (SEQ.ID.NO.:143).

The second round of PCR also utilized caudate nucleus DNA as a template and a 5' PCR primer that contained an EcoRV site with the following sequence:

5'-CCTCGGATATCATGGTCCCAGAGCCGGGCCC-3' (SEQ.ID.NO.:144)

and a 3' PCR primer that contained a XbaI site with the following sequence:

5'-CAGCTCTAGATTGGCCAGCCCCAAGCCCGGGT-3' (SEQ.ID.NO.:145).

Nucleic acid and amino acid sequences were thereafter determined and verified.

5. Dopamine D1

Dopamine D1 was subcloned from a full length cDNA clone obtained from the American Type Culture Collection.

6. Dopamine D2

Dopamine D2 was subcloned from a full length cDNA clone obtained from the American Type Culture Collection.

7. Dopamine D5

To obtain Dopamine D5, PCR was performed using genomic cDNA as template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25μM of each primer, and 0.2 mM of each 4 nucleotides.

Dopamine D5 receptor contained no intron in the coding region. However, Dopamine D5 receptor contained two pseudogenes with 8bp fame shift insertion within the coding region. In order to avoid the pseudogenes, the DNA fragment 5' and 3' of the frame shift insert was each amplified from genomic DNA. The 5' PCR fragment was obtained utilizing the following primer set:

5'-CCTGAATTCCAGCCCGAAATGCTGCCGCCAG-3' (SEQ.ID.NO.:146; sense)

5'-GGTCCACGCTGATGACGCACAGGTTC-3' (SEQ.ID.NO.:147; antisense) and

3' PCR fragment was obtained utilizing the following primer set:

5'-GAACCTGTGCGTCATCAGCGTGGACC-3' (SEQ.ID.NO.: 148; sense)

5'-TGCGGATCCATGAGGGGGTTTCTTAATG-3' (SEQ.ID.NO.:149; antisense).

The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase and SEQ.ID.NO.:146 and SEQ.ID.NO.:149 as primers. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 2 min 30 sec and 72°C for 1 min 30 sec.

The resulting PCR fragment was digested with EcoRI and BamHI restriction sites and cloned into an EcoRI-BamHI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

8. ETA

The endogenous human ETA was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72 °C for 2 sec. The 5' PCR fragment, containing a HindIII site, was obtained utilizing genomic DNA, as a template, and the following primer set:

5'-CGGAAGCTTCTGGAGCAGGTAGCAGCATG-3' (SEQ.ID.NO.:150) and

5'-TGGGCAATAGTTGTGCATTGAGCCA-3'- (SEQ.ID.NO.:151).

The 3' PCR fragment, containing BamHI site, was obtained by pfu polymerase (Stratagene) using IMAGE 666747, as a template, and the following primer set:

5'-CTAATTTGGTCCTACCCAGCAATGGC-3' (SEQ.ID.NO.: 152) and

5'-CTTGGATCCAGATGAGCTGTATTTATTACTGGAACG-3' (SEQ.ID.NO.:153).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 2 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and the following primers:

5'-AATAAGCTTCAAGATGGAAACCCTTTGCCTCAG-3' (SEQ.ID.NO.:154)

5'-CGTTCATGCTGTCCTTATGGCTGCTC-3' (SEQ.ID.NO.:155).

The PCR cycle condition for the full length clone was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min 10 sec.

The resulting PCR fragment was digested with EcoRI restriction site and cloned into an EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

9. mGluR1

The endogenous human mGluR1 was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer. The cycle condition for the first round of PCR was as follows: 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and 50 sec. The 5' PCR fragment contained a SalI site and was obtained utilizing hippocampus DNA as a template, and the following primer set:

5'-GCAGGCTGTCGACCTCGTCCTCACCACCATGGTC-3' (SEQ.ID.NO.:156) and

5'-AATGGGCTCACAGCCTGTTAGATCTGCATTGGGCCAC-3'- (SEQ.ID.NO.:157).

The middle PCR fragment was obtained utilizing genomic DNA as a template, where the cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and 50 sec, with the following primer set:

5'-TAACAGGCTGTGAGCCCATTCCTGTGCG-3' (SEQ.ID.NO.:158) and

5'-TTAGAATTCGCATTCCCTGCCCCTGCCTTCTTTC-3' (SEQ.ID.NO.:159).

The 3' PCR fragment contained a BamHI site and was obtained utilizing genomic cDNA, as a template, and the following primer set:

5'-TGCGAATTCTAATGGCAAGTCTGTGTCATGGTC-3' (SEQ.ID.NO.:160) and

5'-TCCGGATCCCAGGGTGGAAGAGCTTTGCTTGTA-3' (SEQ.ID.NO.:161).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and 15 sec.

The resulting PCR fragment was digested with SalI and BamHI restriction site and cloned into a SalI-BamHI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

10. GPR24 (also known as MCH or SLC-1)

The endogenous human GPR24 was obtained by PCR using genomic DNA as template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 56°C for 1min and 72 °C for 1 min and 20 sec. The 5' PCR primer contained a HindIII site with the sequence:

5'-GTGAAGCTTGCCTCTGGTGCCTGCAGGAGG-3' (SEQ.ID.NO.:162)

and the 3' primer contained an EcoRI site with the sequence:

5'-GCAGAATTCCCGGTGGCGTGTTGTGGTGCCC-3' (SEQ.ID.NO.:163).

The 1.3 kb PCR fragment was digested with HindIII and EcoRI and cloned into HindIII-EcoRI site of CMVp expression vector. Later the cloning work by Lakaye et al showed that there is an intron in the coding region of the gene. Thus the 5' end of the cDNA was obtained by 5' RACE PCR using Clontech's marathon-ready hypothalamus cDNA as template and the manufacturer's recommended protocol for cycling condition. The 5' RACE PCR for the first and second round PCR were as follows:

5'-CATGAGCTGGTGGATCATGAAGGG-3' (SEQ.ID.NO.:164) and

5'-ATGAAGGGCATGCCCAGGAGAAAG-3' (SEQ.ID.NO.:165).

Nucleic acid and amino acid sequences were thereafter determined and verified.

11. NTSR1

The endogenous human NTSR1 was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer, 0.25 μM

of each primer, and 0.2 mM of each 4 nucleotides. The cycle condition was 30 cycles of 94°C for 1 min, 60°C for 1min and 72°C for 1 min and 10 sec. The 5' PCR fragment, containing a HindIII site, was obtained utilizing genomic DNA, as a template, and the following primer set:

5'-CCCAAGCTTCCAGCCCCGGAGGCGCCGGAC-3' (SEQ.ID.NO.: 166) and

5'-TGAAGGTGTTGACCTGTATGACGACCTTGACGGTGGG-3'- (SEQ.ID.NO.:167).

The 3' PCR fragment, containing an EcoRI site, was obtained utilizing brain cDNA, as a template, and the following primer set:

5'-GGTCGTCATACAGGTCAACACCTTCATGTCCTTCATA-3' (SEQ.ID.NO.:168) and

5'-CACGAATTCGTACAGCGTCTCGCGGGTGGCATT-3' (SEQ.ID.NO.:169).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and 10 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and

5'-ATCACCATGCGCCTCAACAGCTCCGC-3' (SEQ.ID.NO.:170)

and SEQ.ID.NO.:169 as primers. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min 10 sec.

The resulting PCR fragment was digested with HindIII and EcoRI restriction site and cloned into a HindIII-EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

12. NTSR2

The endogenous human NTSR2 was obtained by PCR using a template and pfu polymerase (Stratagene) with the buffer system provided by the manufacturer. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1min and 72°C for 1 min and 15 sec.

The 5' PCR fragment was obtained utilizing IMAGE 1537523, as a template, and the following primer set:

5'-ATCACCATGGAAACCAGCAGCCCGCGGC-3' (SEQ.ID.NO.:171) and

5'-CGGGGTAGAAGTGGACGGCACTTGGG-3'- (SEQ.ID.NO.:172).

The 3' PCR fragment, containing an EcoRI site, was obtained utilizing caudate nucleus cDNA, as a template, and the following primer set:

5'-GCTCCCAAGTGCCGTCCACTTCTACC-3' (SEQ.ID.NO.:173) and

5'-TTAGAATTCGGTCCGGGTTTCTGGGGGATCC-3' (SEQ.ID.NO.:174).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and 20 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and SEQ.ID.NO.:171 and SEQ.ID.NO.:174 as primers. The PCR cycle condition for the full length clone was 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min 10 sec.

The resulting PCR fragment was digested with EcoRI restriction site and cloned into an EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

13. OPRM1

The endogenous human OPRM1 was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 40 sec. The 5' PCR fragment, containing a HindIII site, was obtained utilizing genomic DNA as a template, and the following primer set:

5'-CCCAAGCTTCAGTACCATGGACAGCAGCGCTGCC-3' (SEQ.ID.NO.:175) and

5'-CATCTTGGTGTATCTGACAATCACATACATGACCAGGAA-3'- (SEQ.ID.NO.:176).

The middle PCR fragment was obtained utilizing genomic DNA as a template, where the cycle condition was 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 40 sec, and the following primer set:

5'-GTATGTGATTGTCAGATACACCAAGATGAAGACTGCCAC-3' (SEQ.ID.NO.:177) and

5'-TACAATCTATGGAACCTTGCCTGTATTTTGTTGTAGCCA-3' (SEQ.ID.NO.:178).

The 3' PCR fragment was obtained utilizing brain cDNA, as a template, and the following primer set:

5'-CAAAATACAGGCAAGGTTCCATAGATTGTACACTAACAT-3' (SEQ.ID.NO.:179) and

5'-CGGGCAACGGAGCAGTTTCTGCTTCAG-3' (SEQ.ID.NO.:180).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min and 15 sec.

The 5'PCR fragment and the middle PCR fragment were used as templates to obtain the 5'-region through the middle region of OPRM1 ("5'-middle PCR fragment") using the pfu polymerase (Stratagene) and SEQ.ID.NO.:175 and SEQ.ID.NO.:178 with the cycle conditions as follows: 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min and 15 sec.

The 5'-middle PCR fragment and 3' PCR fragment were then used as templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and SEQ.ID.NO.:175 and SEQ.ID.NO.:180 as primers. The cycle condition for the full length PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min 15 sec.

The resulting PCR fragment was digested with HindIII restriction site and cloned into a HindIII pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

14. OPRM1A

The endogenous human OPRM1A was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1min and 72°C for 40 sec. The 5' PCR fragment, containing a HindIII site, was obtained utilizing genomic DNA as a template, and SEQ.ID.NO.:175 and SEQ.ID.NO.:176.

The middle PCR fragment was obtained utilizing genomic DNA as a template, where the cycle condition was 30 cycles of 94°C for 1 min, 60°C for 1min and 72°C for 40 sec, and SEQ.ID.NO.:177 and SEQ.ID.NO.:178.

The 3' PCR fragment was obtained utilizing genomic DNA as a template, and SEQ.ID.NO.:179 and SEQ.ID.NO.:180. The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and 30 sec.

The 5'PCR fragment and the middle PCR fragment were used as co-templates to obtain the 5'-region through the middle region of OPRM1A ("5'-middle PCR fragment") using the pfu polymerase (Stratagene) and SEQ.ID.NO.:175 and SEQ.ID.NO.:178 with the cycle conditions as follows: 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min and 15 sec.

The 5'-middle PCR fragment and 3' PCR fragment were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and SEQ.ID.NO.:175 and SEQ.ID.NO.:180 as primers. The cycle condition for the full length PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min 15 sec.

The resulting PCR fragment was digested with HindIII restriction site and cloned into a HindIII pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

15. OX_1R

The OX_1R EST clone 40608 is a full length cDNA clone. However, it contained a 4bp frame shift insertion. To remove the insert, the fragments 5' and 3' of the frame shift insert was each obtained by PCR using EST clone 40608 as template and two primer pairs. The 5' primer set, containing an EcoRI site, were as follows:

5'-ATGGAATTCTGCTGCAGCGGCTCCTGAGCTC-3' (SEQ. ID.NO.:181; sense)

5'-ACGGACACAGCCTGTAGATAGGGGATGACCTTGCAG-3' (SEQ.ID.NO.:182; antisense)

and the 3' primer set, containing a BamHI site, were as follows:

5'-ATCCCCTATCTACAGGCTGTGTCCGTGTCAGTGGCAG-3' (SEQ.ID.NO.:183; sense)

5'-GGAGGATCCAGGGCAGCCCTCGCTCAGGGC-3' (SEQ.ID.NO.:184; antisense).

The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using SEQ.ID.NO.:181 and SEQ.ID.NO.:184 as primers. The cycle condition for each PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 2 min 30 sec and 72°C for 1 min 30 sec.

The resulting PCR fragment was digested with EcoRI and BamHI restriction sites and cloned into an EcoRI-BamHI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

16. PTHR1

The endogenous human PTHR1 was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer. The cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1min and 72°C for 1 min and 30 sec. The 5' PCR fragment, containing a HindIII site, was obtained utilizing kidney cDNA, as a template, and the following primer set:

5'-CGCAAGCTTAGGCGGTGGCGATGGGGACCGCC -3' (SEQ.ID.NO.:185) and

5'-GGATGTGGTCCCATTCCGGCAGACAG-3'- (SEQ.ID.NO.:186).

The 3' PCR fragment, containing an EcoRI site, was obtained by pfu PCR (Stratagene) and IMAGE 1624048, as a template, and the following primer set:

5'-AGGAGGCACCCACTGGCAGCAGGTA-3' (SEQ.ID.NO.: 187) and

5'-GCCGAATTCCATGACTGTCTCCCACTCTTCCTG-3' (SEQ.ID.NO.:188).

The cycle condition for 3' PCR reaction was as follows: 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and 30 sec. The 5' and 3' PCR fragments were then used as co-templates to obtain the full length cDNA using the pfu polymerase (Stratagene) and SEQ.ID.NO.:185 and SEQ.ID.NO.:188 as primers. The PCR cycle condition for the full length clone was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 3 sec.

The resulting PCR fragment was digested with HindIII and EcoRI restriction site and cloned into a HindIII-EcoRI pCMV expression vector. Nucleic acid and amino acid sequences were thereafter determined and verified.

17. SST2

SST2 was obtained by subcloning EST 06818 into a pCMV vector.

Table E below indicates the GenBank Accession number for which the endogenous receptors set forth above can be located, and for which the endogenous nucleic and amino acid sequences are provided.

TABLE E

Receptor Identifier	GenBank Accession Number
5HT-1A	X13556
5HT-1B	D10995
5HT-1D	M81589
5HT-1E	M91467
5HT-1F	L04962
5HT-2B	X77307
5HT-4A	Y08756
5HT-4B	Y12505

5HT-4C	Y12506
5HT-4D	Y12507
5HT-4E	AJ011371
5HT-5A	X81411
5HT6	L41147
5HT7	L21195
AVPR1A	AF030625
AVPR1B	D31833
AVPR2	NM_000054
BBR3	X76498
BDKR1	AJ238044
BDKR2	NM_000623
C3a	U62027
C5a	M62505
CB1	X54937
CB2	X74328
CCR2b	U03882
CCR3	U28694
CCR5	U54994
CCR8	U45983
CCR9	AJ132337
CRFR1	L23332
CXCR4	AJ224869
Dopamine D1	X55758
Dopamine D2	S62137
Dopamine D3	U32499
Dopamine D5	M67439
ETA	X61950
ETB	L06623
FPR1	M60627
FPRL1	M76672
GALR1	L34339
GALR2	AF040630
GALR3	AF073799
GIP	U39231
mGluR1	L76627
GPR5	L36149
GPR24 (also known as MCH or SLC-1)	U71092
GRPR	M73481
M1	X15263
M2	X15264
M3	X15266
M4	X15265
M5	M80333
MC3	L06155
NK1R	M74290
NK2R	M57414
NK3R	M89473
NMBR	M73482
NPY5	U94320
NTSR1	X70070
NTSR2	Y10148
OPRD	U07882
OPRL1	X77130
OPRK	U11053

OPRM	L25119
OPRM1A	L25119
OX_1R	AF041243
OX_2R	AF041245
PACAP	D17516
PAF	S56396
PGE EP1	L22647
PGE EP2	U19487
PGE EP4	NM_000958
PTHR1	L04308
PTHR2	U25128
SCTR	U28281
SST1	M81829
SST2	M81830
SST3	M96738
SST4	D16826
SST5	D16827
TSHR	AF035261
VIPR	L13288
VIPR2	X95097

Example 2

PREPARATION OF NON-ENDOGENOUS, VERSIONS OF THE KNOWN GPCRS

A. Site-Directed Mutagenesis

Those skilled in the art are credited with the ability to select techniques for mutation of a nucleic acid sequence. Presented below are approaches utilized to create non-endogenous versions of several of the human GPCRs disclosed above. The mutations disclosed below are based upon an algorithmic approach whereby the 16th amino acid (located in the IC3 region of the GPCR) from a conserved proline residue (located in the TM6 region of the GPCR, near the TM6/IC3 interface) is mutated, most preferably to a lysine amino acid residue.

In most of the examples of this Example 2, the algorithmic approach set forth above was used to identify the amino acid residue to be mutated. However, several GPCRs set forth below utilized a modified algorithmic approach (*e.g.*, CRFR1, GIP, mGluR1, GPR24, PTHR1, PTHR2, SCTR, TSHR, VIPR and VIPR2). This modified approach focuses on a conserved proline residue (also located in the TM6 region of the GPCR, near the TM6/IC3

interface) whereby the 5th amino acid upstream from the proline is generally, but not always, a threonine residue. For these receptors, the endogenous 5th amino acid residue is mutated, most preferably to a proline amino acid residue.

Other mutation approaches can be used (*e.g.*, mGluR1, GPR24 and TSHR) and one skilled in the art is credited with the ability to select techniques for mutation of a nucleic acid sequence. The importance here is that the mutation leads to a constitutively activated receptor and given the extension approaches set forth herein for determination of constitutively activity, routine analysis can be employed in this context.

Preparation of non-endogenous known GPCRs is preferably accomplished by using Transformer Site-Directed™ Mutagenesis Kit (Stratagene, according to manufacturer's instructions) or QuikChange Site-Directed™ Mutagenesis (Clontech). Endogenous GPCR is preferably used as a template and two mutagenesis primers utilized, as well as, most preferably, a lysine mutagenesis oligonucleotide and a selection marker oligonucleotide (SEQ.ID.NO.: 252; included in Stratagene's kit). For convenience, the codon mutation incorporated into the known GPCR and the respective oligonucleotides are noted, in standard form (Table F):

TABLE F

Receptor Identifier	Codon Mutation	5'-3' orientation (sense), (SEQ.ID.NO.) mutation underlined	5'-3' orientation (antisense) (SEQ.ID.NO.)
5HT-1A	V343K	CGAGAGAGGAAGACAAAG AAGACGCTGGGCAT (189)	ATGCCCAGCGTCTTCTTT GTCTTCCTCTCTCG (190)
5HT-1B	T313K	GGGAGCGCAAAGCCAAGA AGACCCTAGGGATC (191)	GATCCCTAGGGTCTTCTT GGCTTTGCGCTCCC (192)
5HT-1D	T300K	CGAGAAAGGAAAGCCAAG AAAATCCTGGGCATCATTC (193)	GAATGATGCCCAGGATT TTCTTGGCTTTCCTTTCT CG (194)
5HT-1E	A290K	AGGGAACGGAAGGCAAAA CGCATCCTGGGGCT (195)	AGCCCCAGGATGCGTTT TGCCTTCCGTTCCCT (196)
5HT-1F	A292K	CAAGAGAACGGAAAGCAA AGACTACCCTGGGATTAAT C (197)	GATTAATCCCAGGGTAG TCTTTGCTTTCCGTTCTC TTG (198)

5HT-2B	S323K	AACGAACAGAGAGCCAAA AAGGTCCTAGGGATTG (199)	CAATCCCTAGGACCTTTT TGGCTCTCTGTTCGTT (200)
5HT-4A	A258K	GGACAGAGACCAAAGCAA AGAAGACCCTGTGCATC (201)	GATGCACAGGGTCTTCTT TGCTTTGGTCTCTGTCC (202)
5HT-4B	A258K	GGACAGAGACCAAAGCAA AGAAGACCCTGTGCATC (203)	GATGCACAGGGTCTTCTT TGCTTTGGTCTCTGTCC (204)
5HT-4C	A258K	GGACAGAGACCAAAGCAA AGAAGACCCTGTGCATC (205)	GATGCACAGGGTCTTCTT TGCTTTGGTCTCTGTCC (206)
5HT-4D	A258K	GGACAGAGACCAAAGCAA AGAAGACCCTGTGCATC (207)	GATGCACAGGGTCTTCTT TGCTTTGGTCTCTGTCC (208)
5HT-4E	A258K	GGACAGAGACCAAAGCAA AGAAGACCCTGTGCATC (209)	GATGCACAGGGTCTTCTT TGCTTTGGTCTCTGTCC (210)
5HT-5A	A284K	AAGGAGCAGCGGGCCAAG CTCATGGTGGGCATC (211)	GATGCCCACCATGAGCT TGGCCCGCTGCTCCTT (212)
5HT-6	S267K	CTGAAGGCCAAGCTTACGCT GGGCATCCTGCTGGGCA (213)	ATGCCCAGCGTAAGCTTG GCCTTCAGGGCCTTCCTG CT (214)
5HT-7	A326K	GAACAGAAAGCAAAGACCA CCCTGGGGATCATCGT (215)	CCCAGGGTGGTCTTTGCT TTCTGTTCTCGCTTAAA (216)
AVPR1A	V290K	GCCAAGATCCGCACGAAGA AGATGACTTTTGTGATCG (217)	CGATCACAAAAGTCATCT TCTTCGTGCGGATCTTGG C (218)
AVPR1B	V280K	GGCCAAGATCCGAACAAAG AAGATGACCTTTGTCATC (219)	CGATGACAAAGGTCATCT TCTTTGTTCGGATCTTGG CC (220)
AVPR2	V270K	GCTGTGGCCAAGACTAAGA GGATGACGCTAGTG (221)	CACTAGCGTCATCCTCTT AGTCTTGGCCACAGC (222)
BBR3	270K	CGAAAGAGAATTAAAAGAA CGGTATTGGTGTTG (223)	AATACCGTTCTTTTAATT CTCTTTCGGGATTC (224)
BDKR1	T249K	GCCGCAAGGATAGCAAGAC CAAAGCGCTGATCCTCAC (225)	GTGAGGATCAGCGCTTTG GTCTTGCTATCCTTGCGG C (226)
BDKR2	T269K	CGGAGAGGAGGGCCAAGGT GCTAGTCCTGGT (227)	ACCAGGACTAGCACCTTG GCCCTCCTCTCCG (228)
C3a	F376K	CGCCAAGTCTCAGAGCAAA ACCAAGCGAGTGGCCGTGG TG (229)	CACCACGGCCACTCGCTT GGTTTTGCTCTGAGACTT GGCG (230)
C5a	L241K	CGGTCCACCAAGACAAAGA AGGTGGTGGTGGCA (231)	TGCCACCACCACCTTCTT TGTCTTGGTGGACCG (232)

CB1	A342K	CGCATGGACATTAGGTTAAAGAAGACCCTGGTCCTGA (233)	TCAGGACCAGGGTCTTCTTTAACCTAATGTCCATGCG (234)
CB2	A244K	GGCTGGATGTGAGGTTGAAGAAGACCCTAGGGCTAGTG (235)	CACTAGCCCTAGGGTCTTCTTCAACCTCACATCCAGCC (236)
CCR2b	V242K	GAAGAGGCATAGGGCAAAGAGAGTCATCTTCACC (237)	GGTGAAGATGACTCTCTTTGCCCTATGCCTCTTC (238)
CCR3	I238K	GTAAAAAAAAGTACAAGGCCAAGCGGCTCATTTTTGTCATC (239)	GATGACAAAAATGAGCCGCTTGGCCTTGTACTTTTTTTTAC (240)
CCR5	V234K	GAAGAGGCACAGGGCTAAGAGGCTTATCTTCACCATC (241)	GATGGTGAAGATAAGCCTCTTAGCCCTGTGCCTCTTC (242)
CCR8	I237K	CCACAACAAGACCAAGGCCAAGAGGTTGGTGCTCATTGTGG (243)	CCACAATGAGCACCAACCTCTTGGCCTTGGTCTTGTTGTGG (244)
CCR9	L253K	TCCAAGCACAAAGCCAAAAAAGTGACCATCACTGTCC (245)	GGACAGTGATGGTCACTTTTTTGGCTTTGTGCTTGGA (246)
CRFR1	T316P	GAAGGCTGTGAAAGCCCCTCTGGTGCTGCTGC (247)	GCAGCAGCACCAGAGGGGCTTTCACAGCCTTC (248)
CXCR4	L238K	AGAAGCGCAAGGCCAAGAAGACCACAGTCATCCTCA (249)	TGAGGATGACTGTGGTCTTCTTGGCCTTGCGCTTCT (250)
Dopamine D1	L271K	GAGAAACTAAAGTCAAGAAGACTCTGTG (251)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
Dopamine D2	T372K	GAGAAGAAAGCCAATCAGATGCTCGCC (253)	GGCGAGCATCTGAGTGGCTTTCTTCTC (254)
Dopamine D3	T328K	GAGAAGAAGGCAAAACAAATGGTGGCC (255)	GGCCACCATTTGTTTTGCCTTCTTCTC (256)
Dopamine D5	L295K	AAGAAGGAGACCAAAGTTAAAAAGACCCTGTCG (257)	CGACAGGGTCTTTTTAACTTTGGTCTCCTTCTT (258)
ETA	A305K	CAGCGTCGAGAAGTGAAAAAAACAGTTTTCTGCTTGGTTGTA (259)	TACAACCAAGCAGAAAACTGTTTTTTTCACTTCTCGACGCTG (260)
ETB	A322K	CAGAGACGGGAAGTGAAGAAAACCGTCTTTTGCCTGG (261)	CCAGGCAAAAGACGGTTTTCTTCACTTCCCGTCTCTG (262)
FPR1	L240K	AAGTCCAGTCGTCCCAAACGGGTCCTCTCCTT (263)	AAGGAGAGGACCCGTTTGGGACGACTGGACTT (264)
FPRL1	L240K	AAATCCAGCCGTCCCAAACGGGTCCTCACTGC (265)	GCAGTGAGGACCCGTTTGGGACGGCTGGATTT (266)
GALR1	A246K	CCAAGAAAAAGACTAAACAGACAGTTCTGG (267)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
GALR2	T235K	GCCAAGCGCAAGGTGAAACGCATGATCCTC (268)	GAGGATCATGCGTTTCACCTTGCGCTTGGCG (269)
GIP	T343P	AGGCTGGCTCGCTCCCCGCTGACGCTGGTGC (270)	GCACCAGCGTCAGCGGGGAGCGAGCCAGCCT (271)
mGluR1	3' Deletion	*See alternative approach below*	*See alternative approach below*
GPR5	V224K	CGGCGCCACCGCACGAAAAAGCTCATCTTC (272)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)

GPR24 (also known as MCH or SLC-1)	T255K T255K/T257R 24-IC3-SST2 C305Y P271L W269C W269F W269L F265I I261Q D140N	*See alternative approach below*	*See alternative approach below*
GRPR	A263K	GGAAGCGACTTAAGAAGAC AGTGCTGGTGTTT (273)	CAGCACTGTCTTCTTAAG TCGCTTCCGGGATTC (274)
M1	A364K	AAGGAGAAGAAGGCGAAAC GGACCCTGAGTGCC (275)	GGCACTCAGGGTCCGTTT CGCCTTCTTCTCCTT (276)
M2	T386K	CCGGGAAAAGAAAGTCAAG AGGACAATCTTGGCT (277)	AGCCAAGATTGTCCTCTT GACTTTCTTTTCCCGG (278)
M3	A490K	GGTCAAGGAGAAGAAAGCG AAACAGACCCTCAGTGCG (279)	CGCACTGAGGGTCTGTTT CGCTTTCTTCTCCTTGACC (280)
M4	T399K	GGGAGCGCAAAGTGAAACG AACGATCTTTGCC (281)	GGCAAAGATCGTTCGTTT CACTTTGCGCTCCC (282)
M5	A441K	GTCAAAGAGAGGAAAGCAA AACAGACACTGAGTGCC (283)	GGCACTCAGTGTCTGTTT TGCTTTCCTCTCTTTGAC (284)
MC3	A241K	GCAACACTCATGTATGAAG GGGAAAGTCACCATCACC (285)	CTCCTTCGGTCCTCCTAT CGTTGTCAGAAGT (252)
NK1R	V247K	GCCAAGCGCAAGGTGAAGA AAATGATGATTGTC (286)	GACAATCATCATTTTCTT CACCTTGCGCTTGGC (287)
NK2R	V249K	GCCAAGAAGAAGTTTAAGA AGACCATGGTGCT (288)	AGCACCATGGTCTTCTTA AACTTCTTCTTGGC (289)
NK3R	V298K	GGCCAAAAGAAAGGTTAAG AAAATGATGATTATTG (290)	CAATAATCATCATTTTCT TAACCTTTCTTTTGGCC (291)
NMBR	A265K	CACGGAAACGCCTGAAAAA AATTGTGCTTG (292)	CAAGCACAATTTTTTTCA GGCGTTTCCGTG (293)
NPY5	F367K	GAATAAAAAAGAGATCACG AAGTGTTAAGTACAGACTG ACC (294)	GGTCAGTCTGTACTTAAC ACTTCGTGATCTCTTTTTT AT (295)
NTSR1	V302K	GCCCTGCGGCACGGCAAGC GCGTCCTACGTGC (296)	GCACGTAGGACGCGCTTG CCGTGCCGCAGGGC (297)
NTSR2	V269K	AGCCTCCAGCGCAGCAAGC AGGTTCTCAGAGCC (298)	GGCTCTGAGAACCTGCTT GCTGCGCTGGAGGCT (299)
OPRD	T260K	GCCTGCGGCGCATCAAGCG CATGGTGCTGGT (300)	ACCAGCACCATGCGCTTG ATGCGCCGCAGGC (301)
OPRL1	T262K	ACCTGCGGCGCATCAAGCG GCTGGTGCTGGTG (302)	CACCAGCACCAGCCGCTT GATGCGCCGCAGGT (303)
OPRK	T273K	ACCTGCGTAGGATCAAGAG ACTGGTCCTGGTG (304)	CACCAGGACCAGTCTCTT GATCCTACGCAGGT (305)
OPRM	T281K	GGGAATCTTCGAAGGATCA AGAGGATGGTGCTGGTG (306)	CACCAGCACCATCCTCTT GATCCTTCGAAGATTCC (307)

OPRM1A	T281K	GGGAATCTTCGAAGGATCAAGAGGATGGTGCTGGTG (308)	CACCAGCACCATCCTCTTGATCCTTCGAAGATTCC (309)
OX_1R	A297K	CGGAGGAAGACAAAAAAGATGCTGATGG (310)	CCATCAGCATCTTTTTTGTCTTCCTCCG (311)
OX_2R	A303K	CCAGAAGGAAAACAAAACGGATGTTGATG (312)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
PACAP	T355K	CGACTGGCCCGGTCCCCCTGCTGCTCATCC (313)	GGATGAGCAGCAGGGGGACCGGGCCAGTCG (314)
PAF	L231K	GTCAAGCGCCGGGCGAAGTGGATGGTGTGCAC (315)	GTGCACACCATCCACTTCGCCCGGCGCTTGAC (316)
PGE EP1	V296K	CACGACGTGGAGATGAAGGGCCAGCTTGTCGG (317)	CCGACAAGCTGGCCCTTCATCTCCACGTCGTG (318)
PGE EP2	L263K	GAGGAGACGGACCACAAGATTCTCCTGGCTATCATG (319)	CATGATAGCCAGGAGAATCTTGTGGTCCGTCTCCTC (320)
PGE EP4	V271K	GCCGAGATCCAGATGAAGATCTTACTCATTGCCACC (321)	GGTGGCAATGAGTAAGATCTTCATCTGGATCTCGGC (322)
PTHR1	T410P	GGAAGCTGCTCAAATCCCCGCTGGTGCTCATGC (323)	GCATGAGCACCAGCGGGGATTTGAGCAGCTTCC (324)
PTHR2	T365P	GGAAACTGGCCAAATCGCCACTGGTCCTGGTCC (325)	GGACCAGGACCAGTGGCGATTTGGCCAGTTTCC (326)
SCTR	T344P	CGCCTGGCCAGGTCCCCTCTCCTGCTGATCC (327)	GGATCAGCAGGAGAGGGGACCTGGCCAGGCG (328)
SST1	T270K	CGAGCGCAAGATCAAATTAATGGTGATGG (329)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
SST2	T255K	AGAAGAAGGTCAAACGAATGGTGTCCATCGTG (330)	GGACACCATTCGTTTGACCTTCTTCTCAGACT (331)
SST3	T256K	GAACGCAGGGTCAAGCGCATGGTGGTGGCC (332)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
SST4	T258K	CGGAGAAGAAAATCAAAAGGCTGGTGCTG (333)	CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT (252)
SST5	T247K	TCGGAGCGAAAGGTGAAGCGCATGGTGTTGGTGGT (334)	ACCATGCGCTTCACCTTTCGCTCCGAGCGCCGCCG (335)
TSHR	V509A D619G A623I A623K C672Y D619G/A623K V509A/C672Y V509A/A623K/C672Y	*See alternative approach below*	*See alternative approach below*
VIPR	T343P	AGGCTAGCCAGGTCCCCACTCCTGCTGATCC (336)	GGATCAGCAGGAGTGGGGACCTGGCTAGCCT (337)
VIPR2	T330P	AGGCTGGCCAAGTCCCCGCTCCTGCTTATCC (338)	GGATAAGCAGGAGCGGGGACTTGGCCAGCCT (339)

B. Alternative Approaches To Mutation

1. mGluR1

Preparation of a non-endogenous version of the human mGluR1 receptor was accomplished by deleting a portion of the intracellular region at the 3' end. The non-endogenous human mGluR1 was obtained by PCR using a template and rTth polymerase (Perkin Elmer) with the buffer system provided by the manufacturer. The cycle condition for the first round of PCR was as follows: 30 cycles of 94°C for 1 min, 65°C for 1min and 72°C for 1 min and 50 sec. The 5' PCR fragment contained a SalI site and was obtained utilizing hippocampus DNA as a template, and the following primer set:

5'-GCAGGCTGTCGACCTCGTCCTCACCACCATGGTC-3' (SEQ.ID.NO.:340) and

5'-AATGGGCTCACAGCCTGTTAGATCTGCATTGGGCCAC-3'- (SEQ.ID.NO.:341).

The middle PCR fragment was obtained utilizing genomic DNA as a template, where the cycle condition was 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 1 min and 50 sec, with the following primer set:

5'-TAACAGGCTGTGAGCCCATTCCTGTGCG-3' (SEQ.ID.NO.:342) and

5'-TTAGAATTCGCATTCCCTGCCCCTGCCTTCTTTC-3' (SEQ.ID.NO.:343).

The 3' PCR fragment was obtained by utilizing the endogenous mGluR1 clone as a co-template to obtain the full length cDNA using the pfu polymerase. The cycle condition for this PCR reaction was 30 cycles of 94°C for 1 min, 65°C for 2 min 30 sec and 72°C for 1 min 30 sec. and the following primer set:

5'-TGCGAATTCTAATGGCAAGTCTGTGTCATGGTC-3' (SEQ.ID.NO.:344) and

5'-TGCGGATCCTCTTCGGAAGATGTTGAGGAAAGTG-3' (SEQ.ID.NO.:345).

(*See*, SEQ.ID.NO.:346 for nucleic acid sequence and SEQ.ID.NO.:347 for amino acid sequence).

2. GPR24 (MCH or SLC-1)

Preparation of non-endogenous versions of the human GPR24 receptor was accomplished by creating an T255K mutation (*see*, SEQ.ID.NO.:350 for nucleic acid sequence, SEQ.ID.NO.:351 for amino acid sequence), a T255K/T257R mutation (*see*, SEQ.ID.NO.:354 for nucleic acid sequence, SEQ.ID.NO.:355 for amino acid sequence), an 24-IC3-SST3 mutation (*see*, SEQ.ID.NO.:358 for nucleic acid sequence, SEQ.ID.NO.:359 for amino acid sequence), a C305Y mutation (*see*, SEQ.ID.NO.:362 for nucleic acid sequence, SEQ.ID.NO.:363 for amino acid sequence), a P271L mutation (*see*, SEQ.ID.NO.:366 for nucleic acid sequence, SEQ.ID.NO.:367 for amino acid sequence), a W269C mutation (*see*, SEQ.ID.NO.:370 for nucleic acid sequence, SEQ.ID.NO.:371 for amino acid sequence), a W269F mutation *see*, SEQ.ID.NO.:374 for nucleic acid sequence, SEQ.ID.NO.:375 for amino acid sequence), and a W269L mutation (*see*, SEQ.ID.NO.:378 for nucleic acid sequence, SEQ.ID.NO.:379 for amino acid sequence), a F265I mutation *see*, SEQ.ID.NO.:382 for nucleic acid sequence, SEQ.ID.NO.:383 for amino acid sequence), an I261Q mutation *see*, SEQ.ID.NO.:386 for nucleic acid sequence, SEQ.ID.NO.:387 for amino acid sequence), and a D140N mutation *see*, SEQ.ID.NO.:390 for nucleic acid sequence, SEQ.ID.NO.:391 for amino acid sequence).

A. T255K Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an T255K mutation (*see*, SEQ.ID.NO.:350 for nucleic acid sequence, and SEQ.ID.NO.:351 for amino acid sequence). Mutagenesis was performed using Transformer Site-Directed Mutagenesis Kit (Clontech) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-AGAGGGTGAAACGCACAGCCATCGCCATCTG-3' (SEQ.ID.NO.:348)

and the antisense primer (selection marker) had the following sequence:

5'-CTCCTTCGGTCCTCCTATCGTTGTCAGAAGT -3' (SEQ.ID.NO.:349).

The endogenous GPR24 cDNA was used as a template.

B. T255K/T257R Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an T255K/T257R mutation (*see*, SEQ.ID.NO.:354 for nucleic acid sequence, and SEQ.ID.NO.:355 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-AGAGGGTGAAACGCAGAGCCATCGCCATCTG-3' (SEQ.ID.NO.:352)

and the antisense primer had the following sequence:

5'-CAGATGGCGATGGCTCTGCGTTTCACCCTCT-3' (SEQ.ID.NO.:353).

The endogenous GPR24 cDNA was used as a template.

C. 24-IC3-SST2 Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating a 24-IC3-SST2 mutation (*see;* SEQ.ID.NO.:358 for nucleic acid sequence, and SEQ.ID.NO.:359 for amino acid sequence). Blast result showed that GPR24 had the highest sequence homology to SST2. Thus the IC3 loop of GPR24 was replaced with that of SST2 to see if the chimera would show constitutive activity.

The BamHI-BstEII fragment containing IC3 of GPR24 was replaced with synthetic oligonucleotides that contained the IC3 of SST2. The PCR sense mutagenesis primer used had the following sequence:

5'-GATCCTGCAGAAGGTGAAGTCCTCTGGAATCCGAGTGGGCTCCTCTAAGAGG AAGAAGTCTGAGAAGAAG-3' (SEQ.ID.NO.:356)

and the antisense primer had the following sequence:

5'-GTGACCTTCTTCTCAGACTTCTTCCTCTTAGAGGAGCCCACTCGGATTCCAGAG

GACTTCACCTTCTGCAG-3' (SEQ.ID.NO.:357).

The endogenous GPR24 cDNA was used as a template.

D. C305Y Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an C305Y mutation (*see*, SEQ.ID.NO.:362 for nucleic acid sequence, and SEQ.ID.NO.:363 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'- GGCTATGCCAACAGCTACCTCAACCCCTTTGTG -3' (SEQ.ID.NO.:360)

and the antisense primer had the following sequence:

5'- CACAAAGGGGTTGAGGTAGCTGTTGGCATAGCC -3' (SEQ.ID.NO.:361).

The endogenous GPR24 cDNA was used as a template.

E. P271L Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an P271L mutation (*see*, SEQ.ID.NO.:366 for nucleic acid sequence, and SEQ.ID.NO.:367 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-TTGTGTGCTGGGCACTCTACTATGTGCTACAGC -3' (SEQ.ID.NO.:364)

and the antisense primer had the following sequence:

5'-GCTGTAGCACATAGTAGAGTGCCCAGCACACAA-3' (SEQ.ID.NO.:365).

The endogenous GPR24 cDNA was used as a template.

F. W269C Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an W269C mutation (*see*, SEQ.ID.NO.:370 for nucleic acid sequence, and SEQ.ID.NO.:371 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-GGTCTTCTTTGTGTGCTGCGCACCCTACTATGTG-3' (SEQ.ID.NO.:368)

and the antisense primer had the following sequence:

5'-CACATAGTAGGGTGCGCAGCACACAAAGAAGACC-3' (SEQ.ID.NO.:369).

The endogenous GPR24 cDNA was used as a template.

G. W269F Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an W269F mutation (*see*, SEQ.ID.NO.:374 for nucleic acid sequence, and SEQ.ID.NO.:375 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-GGTCTTCTTTGTGTGCTTCGCACCCTACTATGTG-3' (SEQ.ID.NO.:372)

and the antisense primer had the following sequence:

5'-CACATAGTAGGGTGCGAAGCACACAAAGAAGACC-3' (SEQ.ID.NO.:373).

The endogenous GPR24 cDNA was used as a template.

H. W269L Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an W269L mutation (*see*, SEQ.ID.NO.:378 for nucleic acid sequence, and SEQ.ID.NO.:379 for amino acid sequence). Mutagenesis was performed

using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-GGTCTTCTTTGTGTGCTTGGCACCCTACTATGTG-3' (SEQ.ID.NO.:376)

and the antisense primer had the following sequence:

5'-CACATAGTAGGGTGCCAAGCACACAAAGAAGACC-3' (SEQ.ID.NO.:377).

The endogenous GPR24 cDNA was used as a template.

I. F265I Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an F265I mutation (*see*, SEQ.ID.NO.:382 for nucleic acid sequence, and SEQ.ID.NO.:383 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-GCCATCTGTCTGGTCATCTTTGTGTGCTGGG-3' (SEQ.ID.NO.:380)

and the antisense primer had the following sequence:

5'-CCCAGCACACAAAGATGACCAGACAGATGGC-3' (SEQ.ID.NO.:381).

The endogenous GPR24 cDNA was used as a template.

J. I261Q Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an I261Q mutation (*see*, SEQ.ID.NO.:386 for nucleic acid sequence, and SEQ.ID.NO.:387 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-CGCACAGCCATCGCCCAGTGTCTGGTCTTCTTTGTG-3' (SEQ.ID.NO.:384)

and the antisense primer had the following sequence:

5'-CACAAAGAAGACCAGACACTGGGCGATGGCTGTGCG-3' (SEQ.ID.NO.:385).

The endogenous GPR24 cDNA was used as a template.

K. D140N Mutation

Preparation of a non-endogenous version of the human GPR24 receptor was accomplished by creating an D140N mutation (*see*, SEQ.ID.NO.:390 for nucleic acid sequence, and SEQ.ID.NO.:391 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-ACCGCCATGGCCATTAACGCGTACCTGGCCACT-3' (SEQ.ID.NO.:388)

and the antisense primer had the following sequence:

5'-AGTGGCCAGGTAGCGGTTAATGGCCATGGCGGT-3' (SEQ.ID.NO.:389).

The endogenous GPR24 cDNA was used as a template.

3. TSHR

Preparation of non-endogenous versions of the human TSHR receptor were accomplished by creating an V509A mutation (*see*, SEQ.ID.NO.:394 for nucleic acid sequence, SEQ.ID.NO.:395 for amino acid sequence), a D619G mutation (*see*, SEQ.ID.NO.:398 for nucleic acid sequence, SEQ.ID.NO.:399 for amino acid sequence), an A623I mutation (*see*, SEQ.ID.NO.:402 for nucleic acid sequence, SEQ.ID.NO.:403 for amino acid sequence), a A623K mutation (*see*, SEQ.ID.NO.:406 for nucleic acid sequence, SEQ.ID.NO.:407 for amino acid sequence), an C672Y mutation (*see*, SEQ.ID.NO.:410 for nucleic acid sequence, SEQ.ID.NO.:411 for amino acid sequence), a D619G/A623K mutation (*see*, SEQ.ID.NO.:414 for nucleic acid sequence, SEQ.ID.NO.:415 for amino acid sequence), an V509A/C672Y mutation *see*, SEQ.ID.NO.:418 for nucleic acid sequence,

SEQ.ID.NO.:419 for amino acid sequence), and an V509A/A623K/C672Y mutation (*see*, SEQ.ID.NO.:422 for nucleic acid sequence, SEQ.ID.NO.:423 for amino acid sequence).

A. V509A Mutation

Preparation of a non-endogenous version of the human TSHR receptor was accomplished by creating an V509A mutation (see, SEQ.ID.NO.:394 for nucleic acid sequence, and SEQ.ID.NO.:395 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-CAAGCGAGTTATCGGCATATACGCTGACGGTC-3' (SEQ.ID.NO.:392)

and the antisense primer had the following sequence:

5'-GACCGTCAGCGTATATGCCGATAACTCGCTTG-3' (SEQ.ID.NO.:393).

The endogenous TSHR cDNA was used as a template. This V509A mutant can be differentiated from the endogenous version by the absence of an AccI site near the mutation site.

B. D619G Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a D619G mutation (see, SEQ.ID.NO.:398 for nucleic acid sequence, and SEQ.ID.NO.:399 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-ACCCAGGGGACAAAGGTACCAAAATTGCCAA-3' (SEQ.ID.NO.:396)

and the antisense primer had the following sequence:

5'-TTGGCAATTTTGGTACCTTTGTCCCCTGGGT-3' (SEQ.ID.NO.:397).

The endogenous TSHR cDNA was used as a template. This D619G mutant can be differentiated from the endogenous version by the presence of a KpnI site near the mutation site.

C. A623I Mutation

Preparation of a non-endogenous version of the human TSHR was accomplished by creating an A623I mutation (see, SEQ.ID.NO.:402 for nucleic acid sequence, and SEQ.ID.NO.:403 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-AAAGATACCAAAATTATCAAGAGGATGGCTGT-3' (SEQ.ID.NO.:400)

and the antisense primer had the following sequence:

5'-ACAGCCATCCTCTTGATAATTTTGGTATCTTT-3' (SEQ.ID.NO.:401).

The endogenous TSHR cDNA was used as a template. This A623I mutant can be differentiated from the endogenous version by the absence of a BstXI site near the mutation site.

D. A623K Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a A623K mutation (see, SEQ.ID.NO.:406 for nucleic acid sequence, and SEQ.ID.NO.:407 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'- AAAGATACCAAAATTAAGAAGAGGATGGCTGTG -3' (SEQ.ID.NO.:404)

and the antisense primer had the following sequence:

5'- CACAGCCATCCTCTTCTTAATTTTGGTATCTTT -3' (SEQ.ID.NO.:405).

The endogenous TSHR cDNA was used as a template. This A623K mutant can be differentiated from the endogenous version by the absence of a BstXI site near the mutation site.

E. C672Y Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a C672Y mutation (see, SEQ.ID.NO.:410 for nucleic acid sequence, and SEQ.ID.NO.:411 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-CTATCCACTTAACTCGTACGCCAATCCATTCCTC-3' (SEQ.ID.NO.:408)

and the antisense primer had the following sequence:

5'-GAGGAATGGATTGGCGTACGAGTTAAGTGGATAG-3' (SEQ.ID.NO.:409).

The endogenous TSHR cDNA was used as a template. This C672Y mutant can be differentiated from the endogenous version by the presence of a BsiWI site near the mutation site.

F. D619G/A623K Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a D619G/A623K mutation (see, SEQ.ID.NO.:414for nucleic acid sequence, and SEQ.ID.NO.:415 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The PCR sense mutagenesis primer used had the following sequence:

5'-ACCCAGGGGACAAAGGTACCAAAATTAAGAAGAGGATGGCTGTG-3'

(SEQ.ID.NO.:412)

and the antisense primer had the following sequence:

5'-CACAGCCATCCTCTTCTTAATTTTGGTACCTTTGTCCCCTGGGT-3' (SEQ.ID.NO.:413).

The non-endogenous D619G mutant version of TSHR cDNA was used as a template. This D619G/A623K mutant can be differentiated from the endogenous version by the presence of a KpnI site near the D619G mutation site and absence of a BstXI site near the A623K mutation site.

G. V509A/C672Y Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a V509A/C672Y mutation (see, SEQ.ID.NO.:418 for nucleic acid sequence, and SEQ.ID.NO.:419 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer. The V509A sense mutagenesis primer used had the following sequence:

5'-CAAGCGAGTTATCGGCATATACGCTGACGGTC-3' (SEQ.ID.NO.:416)

and the C672Y antisense primer had the following sequence:

5'-GAGGAATGGATTGGCGTACGAGTTAAGTGGATAG-3' (SEQ.ID.NO.:417).

The endogenous TSHR cDNA was used as a template. This V509A/C672Y mutant can be differentiated from the endogenous version by the absence of an AccI site near the V509A mutation site and presence of a BsiWI site near the C672Y mutation site.

H. V509A/A623K/C672Y Mutation

Preparation of a non-endogenous version of the human TSHR receptor was also accomplished by creating a V509A/A623K/C672Y mutation (see, SEQ.ID.NO.:422 for nucleic acid sequence, and SEQ.ID.NO.:423 for amino acid sequence). Mutagenesis was performed using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to

the manufacturer. The A623K sense mutagenesis primer used had the following sequence:

5'-AAAGATACCAAAATTAAGAAGAGGATGGCTGTG-3' (SEQ.ID.NO.:420)

and the A623K antisense primer had the following sequence:

5'-CACAGCCATCCTCTTCTTAATTTTGGTATCTTT-3' (SEQ.ID.NO.:421).

The non-endogenous V509A/C672Y mutant version of TSHR cDNA was used as a template. This V509A/A623K/C672Y mutant can be differentiated from the endogenous version by the absence of an AccI site near the V509A mutation site, absence of a BstXI near the A623K mutation site and the presence of a BsiWI site near the C672Y mutation site.

The non-endogenous human GPCRs were then sequenced and the derived and verified nucleic acid and amino acid sequences are listed in the accompanying "Sequence Listing" appendix to this patent document, as summarized in Table G below:

Table G

Mutated GPCR	Nucleic Acid Sequence Listing	Amino Acid Sequence Listing
5HT-1A V343K	SEQ.ID.NO.:424	SEQ.ID.NO.:425
5HT-1B T313K	SEQ.ID.NO.:426	SEQ.ID.NO.:427
5HT-1D T300K	SEQ.ID.NO.:428	SEQ.ID.NO.:429
5HT-1E A290K	SEQ.ID.NO.:430	SEQ.ID.NO.:431
5HT-1F A292K	SEQ.ID.NO.:432	SEQ.ID.NO.:433
5HT-2B S323K	SEQ.ID.NO.:434	SEQ.ID.NO.:435
5HT-4A A258K	SEQ.ID.NO.:436	SEQ.ID.NO.:437
5HT-4B A258K	SEQ.ID.NO.:438	SEQ.ID.NO.:439
5HT-4C A258K	SEQ.ID.NO.:440	SEQ.ID.NO.:441
5HT-4D	SEQ.ID.NO.:442	SEQ.ID.NO.:443

A258K		
5HT-4E A258K	SEQ.ID.NO.:444	SEQ.ID.NO.:445
5HT-5A A284K	SEQ.ID.NO.:446	SEQ.ID.NO.:447
5HT-6 S267K	SEQ.ID.NO.:448	SEQ.ID.NO.:449
5HT-7 A326K	SEQ.ID.NO.:450	SEQ.ID.NO.:451
AVPR1A V290K	SEQ.ID.NO.:452	SEQ.ID.NO.:453
AVPR1B V280K	SEQ.ID.NO.:454	SEQ.ID.NO.:455
AVPR2 V270K	SEQ.ID.NO.:456	SEQ.ID.NO.:457
BBR3 A270K	SEQ.ID.NO.:458	SEQ.ID.NO.:459
BDKR1 T249K	SEQ.ID.NO.:460	SEQ.ID.NO.:461
BDKR2 T269K	SEQ.ID.NO.:462	SEQ.ID.NO.:463
C3a F376K	SEQ.ID.NO.:464	SEQ.ID.NO.:465
C5a L241K	SEQ.ID.NO.:466	SEQ.ID.NO.:467
CB1 A342K	SEQ.ID.NO.:468	SEQ.ID.NO.:469
CB2 A244K	SEQ.ID.NO.:470	SEQ.ID.NO.:471
CCR2b V242K	SEQ.ID.NO.:472	SEQ.ID.NO.:473
CCR3 I238K	SEQ.ID.NO.:474	SEQ.ID.NO.:475
CCR5 V234K	SEQ.ID.NO.:476	SEQ.ID.NO.:477
CCR8 I237K	SEQ.ID.NO.:478	SEQ.ID.NO.:479
CCR9 L253K	SEQ.ID.NO.:480	SEQ.ID.NO.:481
CRFR1 T316P	SEQ.ID.NO.:482	SEQ.ID.NO.:483
CXCR4 L238K	SEQ.ID.NO.:484	SEQ.ID.NO.:485
Dopamine D1 L271K	SEQ.ID.NO.:486	SEQ.ID.NO.:487
Dopamine D2 T372K	SEQ.ID.NO.:488	SEQ.ID.NO.:489
Dopamine D3 T328K	SEQ.ID.NO.:490	SEQ.ID.NO.:491
Dopamine D5 L295K	SEQ.ID.NO.:492	SEQ.ID.NO.:493
ETA A305K	SEQ.ID.NO.:494	SEQ.ID.NO.:495
ETB A322K	SEQ.ID.NO.:496	SEQ.ID.NO.:497
FPR1	SEQ.ID.NO.:498	SEQ.ID.NO.:499

L240K		
FPRL1 L240K	SEQ.ID.NO.:500	SEQ.ID.NO.:501
GALR1 A246K	SEQ.ID.NO.:502	SEQ.ID.NO.:503
GALR2 T235K	SEQ.ID.NO.:504	SEQ.ID.NO.:505
GIP T343P	SEQ.ID.NO.:506	SEQ.ID.NO.:507
mGluR1 3' Deletion	SEQ.ID.NO.:346	SEQ.ID.NO.:347
GPR5 V224K	SEQ.ID.NO.:508	SEQ.ID.NO.:509
GPR24 (also known as MCH or SLC-1)		
T255K	SEQ.ID.NO.:350	SEQ.ID.NO.:351
T255K/T257R	SEQ.ID.NO.:354	SEQ.ID.NO.:355
24-IC3-SST2	SEQ.ID.NO.:358	SEQ.ID.NO.:359
C305Y	SEQ.ID.NO.:362	SEQ.ID.NO.:363
P271L	SEQ.ID.NO.:366	SEQ.ID.NO.:367
W269C	SEQ.ID.NO.:370	SEQ.ID.NO.:371
W269F	SEQ.ID.NO.:374	SEQ.ID.NO.:375
W269L	SEQ.ID.NO.:378	SEQ.ID.NO.:379
F265I	SEQ.ID.NO.:382	SEQ.ID.NO.:383
I261Q	SEQ.ID.NO.:386	SEQ.ID.NO.:387
D140N	SEQ.ID.NO.:390	SEQ.ID.NO.:391
GRPR A263K	SEQ.ID.NO.:510	SEQ.ID.NO.:511
M1 A364K	SEQ.ID.NO.:512	SEQ.ID.NO.:513
M2 T386K	SEQ.ID.NO.:514	SEQ.ID.NO.:515
M3 A490K	SEQ.ID.NO.:516	SEQ.ID.NO.:517
M4 T399K	SEQ.ID.NO.:518	SEQ.ID.NO.:519
M5 A441K	SEQ.ID.NO.:520	SEQ.ID.NO.:521
MC3 A241K	SEQ.ID.NO.:522	SEQ.ID.NO.:523
NK1R V247K	SEQ.ID.NO.:524	SEQ.ID.NO.:525
NK2R V249K	SEQ.ID.NO.:526	SEQ.ID.NO.:527
NK3R V298K	SEQ.ID.NO.:528	SEQ.ID.NO.:529
NMBR A265K	SEQ.ID.NO.:530	SEQ.ID.NO.:531
NPY5 A297K	SEQ.ID.NO.:532	SEQ.ID.NO.:533
NTSR1 V302K	SEQ.ID.NO.:534	SEQ.ID.NO.:535
NTSR2 V269K	SEQ.ID.NO.:536	SEQ.ID.NO.:537
OPRD T260K	SEQ.ID.NO.:538	SEQ.ID.NO.:539

OPRL1 T262K	SEQ.ID.NO.:540	SEQ.ID.NO.:541
OPRK T273K	SEQ.ID.NO.:542	SEQ.ID.NO.:543
OPRM T281K	SEQ.ID.NO.:544	SEQ.ID.NO.:545
OPRM1A T281K	SEQ.ID.NO.:546	SEQ.ID.NO.:547
OX_1R F367K	SEQ.ID.NO.:548	SEQ.ID.NO.:549
OX_2R A297K	SEQ.ID.NO.:550	SEQ.ID.NO.:551
PACAP T355K	SEQ.ID.NO.:552	SEQ.ID.NO.:553
PAF L231K	SEQ.ID.NO.:554	SEQ.ID.NO.:555
PGE EP1 V296K	SEQ.ID.NO.:556	SEQ.ID.NO.:557
PGE EP2 L263K	SEQ.ID.NO.:558	SEQ.ID.NO.:559
PGE EP4 V271K	SEQ.ID.NO.:560	SEQ.ID.NO.:561
PTHR1 T410P	SEQ.ID.NO.:562	SEQ.ID.NO.:563
PTHR2 T365P	SEQ.ID.NO.:564	SEQ.ID.NO.:565
SCTR T344P	SEQ.ID.NO.:566	SEQ.ID.NO.:567
SST1 T290K	SEQ.ID.NO.:568	SEQ.ID.NO.:569
SST2 T255K	SEQ.ID.NO.:570	SEQ.ID.NO.:571
SST3 T256K	SEQ.ID.NO.:572	SEQ.ID.NO.:573
SST4 T258K	SEQ.ID.NO.:574	SEQ.ID.NO.:575
SST5 T247K	SEQ.ID.NO.:576	SEQ.ID.NO.:577
TSHR		
V509A	SEQ.ID.NO.:394	SEQ.ID.NO.:395
D619G	SEQ.ID.NO.:398	SEQ.ID.NO.:399
A623I	SEQ.ID.NO.:402	SEQ.ID.NO.:403
A623K	SEQ.ID.NO.:406	SEQ.ID.NO.:407
C672Y	SEQ.ID.NO.:410	SEQ.ID.NO.:411
D619G/A623K	SEQ.ID.NO.:414	SEQ.ID.NO.:415
V509A/C672Y	SEQ.ID.NO.:418	SEQ.ID.NO.:419
V509A/A623K/C672Y	SEQ.ID.NO.:422	SEQ.ID.NO.:423
VIPR T343P	SEQ.ID.NO.:578	SEQ.ID.NO.:579
VIPR2 T330P	SEQ.ID.NO.:580	SEQ.ID.NO.:581

Assessment of constitutive activity of the non-endogenous versions of the known GPCRs can then be accomplished.

Example 3
RECEPTOR EXPRESSION

Although a variety of cells are available to the art for the expression of proteins, it is most preferred that mammalian cells be utilized. The primary reason for this is predicated upon practicalities, *i.e.,* utilization of, *e.g.,* yeast cells for the expression of a GPCR, while possible, introduces into the protocol a non-mammalian cell which may not (indeed, in the case of yeast, does not) include the receptor-coupling, genetic-mechanism and secretary pathways that have evolved for mammalian systems – thus, results obtained in non-mammalian cells, while of potential use, are not as preferred as that obtained from mammalian cells. Of the mammalian cells, COS-7, Hek-293 and Hek-293T cells are particularly preferred, although the specific mammalian cell utilized can be predicated upon the particular needs of the artisan. The following approach was used for the indicated receptors, and can also be applied with respect to other receptors disclosed herein.

On day one, $2X10^4$ Hek-293T cells well were plated out. On day two, two reaction tubes were prepared (the proportions to follow for each tube are per plate): tube A was prepared by mixing 20μg DNA (*e.g.*, pCMV vector; pCMV vector with receptor cDNA, etc.) in 1.2ml serum free DMEM (Irvine Scientific, Irvine, CA); tube B was prepared by mixing 120μl lipofectamine (Gibco BRL) in 1.2ml serum free DMEM. Tubes A and B were admixed by inversions (several times), followed by incubation at room temperature for 30-45min. The admixture is referred to as the "transfection mixture". Plated Hek-293T cells were washed with 1XPBS, followed by addition of 10ml serum free DMEM. 2.4ml of the transfection mixture were added to the cells, followed by incubation for 4hrs at 37°C/5% CO_2. The transfection mixture was removed by aspiration, followed by the

addition of 25ml of DMEM/10% Fetal Bovine Serum. Cells were incubated at 37°C/5% CO_2. After 72hr incubation, cells were harvested and utilized for analysis.

Example 4
ASSAYS FOR DETERMINATION OF CONSTITUTIVE ACTIVITY OF NON-ENDOGENOUS GPCRS

A variety of approaches are available for assessment of constitutive activity of the non-endogenous versions of known GPCRs. The following are illustrative; those of ordinary skill in the art are credited with the ability to determine those techniques that are preferentially beneficial for the needs of the artisan.

1. Membrane Binding Assays: [^{35}S]GTPγS Assay

When a G protein-coupled receptor is in its active state, either as a result of ligand binding or constitutive activation, the receptor couples to a G protein and stimulates the release of GDP and subsequent binding of GTP to the G protein. The alpha subunit of the G protein-receptor complex acts as a GTPase and slowly hydrolyzes the GTP to GDP, at which point the receptor normally is deactivated. Constitutively activated receptors continue to exchange GDP for GTP. The non-hydrolyzable GTP analog, [^{35}S]GTPγS, can be utilized to demonstrate enhanced binding of [^{35}S]GTPγS to membranes expressing constitutively activated receptors. The advantage of using [^{35}S]GTPγS binding to measure constitutive activation is that: (a) it is generically applicable to all G protein-coupled receptors; (b) it is proximal at the membrane surface making it less likely to pick-up molecules which affect the intracellular cascade.

The assay utilizes the ability of G protein coupled receptors to stimulate [^{35}S]GTPγS binding to membranes expressing the relevant receptors. The assay can, therefore, be used in the direct identification method to screen candidate compounds to known, and

constitutively activated G protein-coupled receptors. The assay is generic and has application to drug discovery at all G protein-coupled receptors.

The [^{35}S]GTPγS assay can be incubated in 20 mM HEPES and between 1 and about 20mM $MgCl_2$ (this amount can be adjusted for optimization of results, although 20mM is preferred) pH 7.4, binding buffer with between about 0.3 and about 1.2 nM [^{35}S]GTPγS (this amount can be adjusted for optimization of results, although 1.2 is preferred) and 12.5 to 75 μg membrane protein (*e.g,* COS-7 cells expressing the receptor; this amount can be adjusted for optimization, although 75μg is preferred) and 1 μM GDP (this amount can be changed for optimization) for 1 hour. Wheatgerm agglutinin beads (25 μl; Amersham) should then be added and the mixture incubated for another 30 minutes at room temperature. The tubes are then centrifuged at 1500 x g for 5 minutes at room temperature and then counted in a scintillation counter.

A less costly but equally applicable alternative has been identified which also meets the needs of large scale screening. Flash plates™ and Wallac™ scintistrips may be utilized to format a high throughput [^{35}S]GTPγS binding assay. Furthermore, using this technique, the assay can be utilized for known GPCRs to simultaneously monitor tritiated ligand binding to the receptor at the same time as monitoring the efficacy via [^{35}S]GTPγS binding. This is possible because the Wallac beta counter can switch energy windows to look at both tritium and ^{35}S-labeled probes. This assay may also be used to detect other types of membrane activation events resulting in receptor activation. For example, the assay may be used to monitor ^{32}P phosphorylation of a variety of receptors (both G protein coupled and tyrosine kinase receptors). When the membranes are centrifuged to the bottom of the well, the bound [^{35}S]GTPγS or the ^{32}P-phosphorylated receptor will activate the scintillant which

is coated of the wells. Scinti® strips (Wallac) have been used to demonstrate this principle. In addition, the assay also has utility for measuring ligand binding to receptors using radioactively labeled ligands. In a similar manner, when the radiolabeled bound ligand is centrifuged to the bottom of the well, the scintistrip label comes into proximity with the radiolabeled ligand resulting in activation and detection.

2. Membrane-Based cAMP

A Flash Plate™ Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) designed for cell-based assays can be modified for use with crude plasma membranes. The Flash Plate wells contain a scintillant coating which also contains a specific antibody recognizing cAMP. The cAMP generated in the wells was quantitated by a direct competition for binding of radioactive cAMP tracer to the cAMP antibody. The following serves as a brief protocol for the measurement of changes in cAMP levels in membranes that express the receptors.

Transfected cells are harvested approximately three days after transfection. Membranes were prepared by homogenization of suspended cells in buffer containing 20mM HEPES, pH 7.4 and 10mM $MgCl_2$. Homogenization is performed on ice using a Brinkman Polytron™ for approximately 10 seconds. The resulting homogenate is centrifuged at 49,000 X g for 15 minutes at 4°C. The resulting pellet is then resuspended in buffer containing 20mM HEPES, pH 7.4 and 0.1 mM EDTA, homogenized for 10 seconds, followed by centrifugation at 49,000 X g for 15 minutes at 4°C. The resulting pellet can be stored at -80°C until utilized. On the day of measurement, the membrane pellet is slowly thawed at room temperature, resuspended in buffer containing 20mM HEPES, pH 7.4 and 10mM $MgCl_2$ (these amounts can be optimized, although the values listed herein are

preferred), to yield a final protein concentration of 0.60mg/ml (the resuspended membranes were placed on ice until use).

cAMP standards and Detection Buffer (comprising 2 μCi of tracer [^{125}I cAMP (100 μl)] to 11 ml Detection Buffer) are prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer is prepared fresh for screening and contained 20mM HEPES, pH 7.4, 10mM $MgCl_2$, 20mM (Sigma), 0.1 units/ml creatine phosphokinase (Sigma), 50 μM GTP (Sigma), and 0.2 mM ATP (Sigma); Assay Buffer can be stored on ice until utilized. The assay is initiated by the addition of 50μL of assay buffer followed by addition of 50 μL of membrane suspension to the NEN Flash Plate. The resultant assay mixture is incubated for 60 minutes at room temperature followed by addition of 100 μL of detection buffer. Plates are then incubated an additional 2-4 hours followed by counting in a Wallac MicroBeta™ scintillation counter. Values of cAMP/well are extrapolated from a standard cAMP curve that is contained within each assay plate.

3. Cell-Based cAMP for Gi Coupled Target GPCRs

TSHR is a Gs coupled GPCR that causes the accumulation of cAMP upon activation. TSHR was constitutively activated by mutating amino acid residue 623 (*i.e.*, changing an alanine residue to an isoleucine residue). *See*, SEQ.ID.NO.:402 for nucleic acid sequence and SEQ.ID.NO.:403 for deduced amino acid sequence. A Gi coupled receptor is expected to inhibit adenylyl cyclase, and, therefore, decrease the level of cAMP production, which can make assessment of cAMP levels challenging. An effective technique for measuring the decrease in production of cAMP as an indication of constitutive activation of a Gi coupled receptor can be accomplished by co-transfecting, most preferably, non-endogenous, constitutively activated TSHR (TSHR-A623I) (or an endogenous, constitutively active Gs coupled receptor) as a "signal enhancer" with a Gi

linked target GPCR, such as GPR24, to establish a baseline level of cAMP. Upon creating a non-endogenous version of the Gi coupled receptor, this non-endogenous version of the target GPCR is then co-transfected with the signal enhancer, and it is this material that can be used for screening. We utilized such approach to effectively generate a signal when a cAMP assay is used; this approach is preferably used in the direct identification of candidate compounds against Gi coupled receptors. It is noted that for a Gi coupled GPCR, when this approach is used, an inverse agonist of the target GPCR will increase the cAMP signal and an agonist will decrease the cAMP signal.

On day one, $2X10^4$ Hek-293 and Hek-293T cells/well were plated out. On day two, two reaction tubes were prepared (the proportions to follow for each tube are per plate): tube A was prepared by mixing 2μg DNA of each receptor transfected into the mammalian cells, for a total of 4μg DNA (*e.g.*, pCMV vector; pCMV vector with mutated THSR (TSHR-A623I); TSHR-A623I and GPR24, etc.) in 1.2ml serum free DMEM (Irvine Scientific, Irvine, CA); tube B was prepared by mixing 120µl lipofectamine (Gibco BRL) in 1.2ml serum free DMEM. Tubes A and B were then admixed by inversion (several times), followed by incubation at room temperature for 30-45min. The admixture is referred to as the "transfection mixture". Plated Hek-293 cells were washed with 1XPBS, followed by addition of 10ml serum free DMEM. 2.4ml of the transfection mixture was then added to the cells, followed by incubation for 4hrs at 37°C/5% CO_2. The transfection mixture was then removed by aspiration, followed by the addition of 25ml of DMEM/10% Fetal Bovine Serum. Cells were then incubated at 37°C/5% CO_2. After 24hr incubation, cells were then harvested and utilized for analysis.

A Flash Plate™ Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) designed for cell-based assays can be modified for use with crude plasma membranes. The

Flash Plate wells can contain a scintillant coating which also contains a specific antibody recognizing cAMP. The cAMP generated in the wells can be quantitated by a direct competition for binding of radioactive cAMP tracer to the cAMP antibody. The following serves as a brief protocol for the measurement of changes in cAMP levels in whole cells that express the receptors.

Transfected cells were harvested approximately twenty four hours after transient transfection. Media was carefully aspirated and discarded. Ten milliliters of PBS was gently added to each dish of cells followed by careful aspiration. One milliliter of Sigma cell dissociation buffer and 3ml of PBS are added to each plate. Cells were pipetted off the plate and the cell suspension is collected into a 50ml conical centrifuge tube. Cells were then centrifuged at room temperature at 1,100 rpm for 5 min. The cell pellet was carefully re-suspended into an appropriate volume of PBS (about 3ml/plate). The cells were then counted using a hemocytometer and additional PBS is added to give the appropriate number of cells (with a final concentration of about 50 μl/well).

cAMP standards and Detection Buffer (comprising 1 μCi of tracer [^{125}I cAMP (50 μl] to 11 ml Detection Buffer) was prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer should be prepared fresh for screening and contained 50μL of Stimulation Buffer, 3μL of test compound (12μM final assay concentration) and 50 μL cells, Assay Buffer can be stored on ice until utilized. The assay can be initiated by addition of 50μL of cAMP standards to appropriate wells followed by addition of 50 μL of PBSA to wells H-11 and H12. Fifty μL of Stimulation Buffer was added to all wells. Selected compounds (*e.g.*, TSH, 100nM MCH, MCH/TSH) were added to appropriate wells using a pin tool capable of dispensing 3 μL of compound solution, with a final assay concentration of 12μM test compound and 100 μL total assay volume. The

cells were then added to the wells and incubated for 60 min at room temperature. 100μL of Detection Mix containing tracer cAMP was then added to the wells. Plates were then incubated additional 2 hours followed by counting in a Wallac MicroBeta scintillation counter. Values of cAMP/well were then extrapolated from a standard cAMP curve which is contained within each assay plate.

Figure 1 evidences about a 22% decrease in cAMP production of cells co-transfected with TSHR-A623I (in the presence of TSH) and non-endogenous, constitutively activated GPR24 ("24-IC3-SST2") (262.266 pmol cAMP/well) compared to TSHR-A623I with endogenous GPR24 ("GPR24 wt") (336.50293 pmol cAMP/well). Co-transfection of TSHR-A623I with non-endogenous, constitutively activated GPR24 ("I261Q") evidences about a 27% decrease in production of cAMP when compared to "GPR24 wt." Such a decrease in cAMP production signifies that non-endogenous version of GPR24 ("I261Q") is constitutively active. Thus, a candidate compound which impacts the GPR24 receptor by increasing the cAMP signal is an inverse agonist, while a GPR24 agonist will decrease the cAMP signal. Based upon the data generated for Figure 1, 24-IC3-SST2 and I261Q are most preferred non-endogenous versions of GPR24 when used in a TSHR (constitutively activated co-transfection approach using a cAMP assay.

Figure 2 evidences about a 60% decrease in cAMP production of cells co-transfected with TSHR-A623I (in the presence of TSH) and non-endogenous, constitutively activated GPR5 ("V224K") (23.5 pmole cAMP/well) compared to TSHR-A623I with endogenous GPR5 ("GPR5 wt") (58.79 pmol cAMP/well). About a 78% and about a 45% decrease in production of cAMP was evidenced when comparing TSHR-A623I co-transfected with "V225K" and TSHR-A623I co-transfected with "GPR5 wt" against pCMV co-transfected with TSHR-A623I (106.75 pmol cAMP/well), respectively. As mentioned

above, a decrease in cAMP production evidences a constitutively active GPR5. Thus, a preferred candidate compound (*i.e.*, an inverse agonist) would likely bind the Gi coupled receptor to increase the signal of activation.

Preferably, and as noted previously, to ensure that a small molecule candidate compound is targeting the Gi coupled target receptor and not, for example, the TSHR-A623I, the directly identified candidate compound is preferably screened against the signal enhancer in the absence of the target receptor.

C. Reporter-Based Assays

1. CRE-Luc Reporter Assay (Gs-associated receptors)

A method to detect Gs stimulation depends on the known property of the transcription factor CREB, which is activated in a cAMP-dependent manner. A PathDetect™ CREB trans-Reporting System (Stratagene, Catalogue # 219010) can utilized to assay for Gs coupled activity in 293 or 293T cells. Cells are transfected with the plasmids components of this above system and the indicated expression plasmid encoding endogenous or mutant receptor using a Mammalian Transfection Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 400 ng pFR-Luc (luciferase reporter plasmid containing Gal4 recognition sequences), 40 ng pFA2-CREB (Gal4-CREB fusion protein containing the Gal4 DNA-binding domain), 80 ng pCMV-receptor expression plasmid (comprising the receptor) and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate per the Kit's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells overnight, and replaced with fresh medium the following

morning. Forty-eight (48) hr after the start of the transfection, cells are treated and assayed for, *e.g.*, luciferase activity

2. 8XCRE-LUC Reporter Assay

HEK-293T cells are plated-out on 96 well plates at a density of 3 x 10^4 cells per well and were transfected using Lipofectamine Reagent (BRL) the following day according to manufacturer instructions. A DNA/lipid mixture is prepared for each 6-well transfection as follows: 260ng of plasmid DNA in 100µl of DMEM were gently mixed with 2µl of lipid in 100µl of DMEM (the 260ng of plasmid DNA consisted of 200ng of a 8xCRE-Luc reporter plasmid (*see* below and Figure 1 for a representation of a portion of the plasmid), 50ng of pCMV comprising endogenous receptor or non-endogenous receptor or pCMV alone, and 10ng of a GPRS expression plasmid (GPRS in pcDNA3 (Invitrogen)). The 8XCRE-Luc reporter plasmid was prepared as follows: vector SRIF-β-gal was obtained by cloning the rat somatostatin promoter (-71/+51) at BglV-HindIII site in the pβgal-Basic Vector (Clontech). Eight (8) copies of cAMP response element were obtained by PCR from an adenovirus template AdpCF126CCRE8 (*see*, 7 *Human Gene Therapy* 1883 (1996)) and cloned into the SRIF-β-gal vector at the Kpn-BglV site, resulting in the 8xCRE-β-gal reporter vector. The 8xCRE-Luc reporter plasmid was generated by replacing the beta-galactosidase gene in the 8xCRE-β-gal reporter vector with the luciferase gene obtained from the pGL3-basic vector (Promega) at the HindIII-BamHI site. Following 30 min. incubation at room temperature, the DNA/lipid mixture was diluted with 400 µl of DMEM and 100µl of the diluted mixture was added to each well. 100 µl of DMEM with 10% FCS were added to each well after a 4hr incubation in a cell culture incubator. The following day the transfected cells were changed with 200

µl/well of DMEM with 10% FCS. Eight (8) hours later, the wells were changed to 100 µl /well of DMEM without phenol red, after one wash with PBS. Luciferase activity were measured the next day using the LucLite™ reporter gene assay kit (Packard) following manufacturer instructions and read on a 1450 MicroBeta™ scintillation and luminescence counter (Wallac).

Figure 3A represents about a 63% increase in activity of the non-endogenous, constitutively active version of human Dopamine D1 receptor (189270 relative light units) compared with that of the endogenous Dopamine D1 (70622 relative light units).

Figure 3B represents about a 48% decreases in activity of the non-endogenous, constitutively active version of human OPRM (a Gi coupled receptor; *see* Example 4(3)), about a 53% decrease in activity of the non-endogenous, constitutively active version of human 5-HT1A (a Gi coupled receptor; *see* Example 4(3)), about a 91% increase in activity of the non-endogenous, constitutively active version of human 5-HT1B, and about a 20% increase in activity of the non-endogenous, constitutively active version of human 5-HT2B over the respective endogenous version of the GPCR.

Figure 3C represents about a 29% increase in activity of the non-endogenous, constitutively active version of human CCR3, about a 41% increase in activity of the non-endogenous, constitutively active version of human NTSR1, about a 51% increase in activity of the non-endogenous, constitutively active version of human CB2, and about a 40% decrease in activity of the non-endogenous, constitutively active version of human CXCR4 (a Gi coupled receptor; *see* Example 4(3)) over the respective endogenous version of the GPCR.

Figure 3D represents about a 75% increase in activity of the non-endogenous, constitutively active version of human PTHR1, about a 74% increase in activity of the

non-endogenous, constitutively active version of human PTHR2, about a 56% increase in activity of the non-endogenous, constitutively active version of human SCTR, about a 96% increase in activity of the non-endogenous, constitutively active version of human PACAP, about a 88% increase in activity of the non-endogenous, constitutively active version of human VIPR1, and about a 91% increase in activity of the non-endogenous, constitutively active version of human VIPR2 over the respective endogenous version of the GPCR.

Figure 3E represents about a 51% increase in activity of the non-endogenous, constitutively active version of human NTSR1, about a 31% decrease in activity of the non-endogenous, constitutively active version of human M1, about a 19% decrease in activity of the non-endogenous, constitutively active version of human M2, about a 32% increase in activity of the non-endogenous, constitutively active version of human M3, about a 33% decrease in activity of the non-endogenous, constitutively active version of human M4, about a 17% decrease in activity of the non-endogenous, constitutively active version of human M5, and about a 60% increase in activity of the non-endogenous, constitutively active version of human 5-HT1D over the respective endogenous version of the GPCR. M2, M4 and 5-HT1D are indicated as being Gi coupled while NTSR1, M1, M3 and M5 are indicated as being Gq coupled.

3. AP1 reporter assay (Gq-associated receptors)

A method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing AP1 elements in their promoter. A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalog # 219073) can be utilized following the protocol set forth above with respect to the CREB

reporter assay, except that the components of the calcium phosphate precipitate were 410 ng pAP1-Luc, 80 ng pCMV-receptor expression plasmid, and 20 ng CMV-SEAP.

4. SRF-LUC Reporter Assay (Gq-associated receptors)

One method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing serum response factors in their promoter. A Pathdetect™ SRF-Luc-Reporting System (Stratagene) can be utilized to assay for Gq coupled activity in, *e.g.*, COS7 cells. Cells are transfected with the plasmid components of the system and the indicated expression plasmid encoding endogenous or non-endogenous GPCR using a Mammalian Transfection™ Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 410 ng SRF-Luc, 80 ng pCMV-receptor expression plasmid and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate as per the manufacturer's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells in a serum free media for 24 hours. The last 5 hours the cells are incubated with 1μM Angiotensin, where indicated. Cells are then lysed and assayed for luciferase activity using a Luclite™ Kit (Packard, Cat. # 6016911) and "Trilux 1450 Microbeta" liquid scintillation and luminescence counter (Wallac) per the manufacturer's instructions. The data can be analyzed using GraphPad Prism™ 2.0a (GraphPad Software Inc.).

5. Intracellular IP_3 Accumulation Assay (Gq-associated receptors)

On day 1, cells comprising the receptors (endogenous and/or non-endogenous) can be plated onto 24 well plates, usually $1x10^5$ cells/well (although this number can be

optimized. On day 2 cells can be transfected by firstly mixing 0.25μg DNA in 50 μL serum free DMEM/well and 2 μL lipofectamine in 50 μl serum-free DMEM/well. The solutions are gently mixed and incubated for 15-30 min at room temperature. Cells are washed with 0.5 ml PBS and 400 μl of serum free media is mixed with the transfection media and added to the cells. The cells are then incubated for 3-4 hrs at 37°C/5%CO_2 and then the transfection media is removed and replaced with 1ml/well of regular growth media. On day 3 the cells are labeled with ^{3}H-myo-inositol. Briefly, the media is removed and the cells are washed with 0.5 ml PBS. Then 0.5 ml inositol-free/serum free media (GIBCO BRL) is added/well with 0.25 μCi of ^{3}H-myo-inositol/ well and the cells are incubated for 16-18 hrs o/n at 37°C/5%CO_2 . On Day 4 the cells are washed with 0.5 ml PBS and 0.45 ml of assay medium is added containing inositol-free/serum free media 10μM pargyline 10 mM lithium chloride or 0.4 ml of assay medium and 50 μL of 10x ketanserin (ket) to final concentration of 10μM. The cells are then incubated for 30 min at 37°C. The cells are then washed with 0.5 ml PBS and 200 μL of fresh/ice cold stop solution (1M KOH; 18 mM Na-borate; 3.8 mM EDTA) is added/well. The solution is kept on ice for 5-10 min or until cells were lysed and then neutralized by 200 μl of fresh/ice cold neutralization sol. (7.5 % HCL). The lysate is then transferred into 1.5 ml eppendorf tubes and 1 ml of chloroform/methanol (1:2) is added/tube. The solution is vortexed for 15 sec and the upper phase is applied to a Biorad AG1-X8™ anion exchange resin (100-200 mesh). Firstly, the resin is washed with water at 1:1.25 W/V and 0.9 ml of upper phase is loaded onto the column. The column is washed with 10 mls of 5 mM myo-inositol and 10 ml of 5 mM Na-borate/60mM Na-formate. The inositol tris phosphates are eluted into scintillation vials containing 10 ml of scintillation cocktail with 2 ml of 0.1 M formic acid/ 1 M ammonium formate. The columns are

regenerated by washing with 10 ml of 0.1 M formic acid/3M ammonium formate and rinsed twice with H_2O and stored at 4°C in water.

Figure 4 represents two preferred non-endogenous, constitutively activated exemplary versions of GPR24, 24-IC3-SST2 and I261Q, for use in an IP3 assay. When compared to the endogenous version of GPR24 ("GPR24 wt"), 24-IC3-SST2 evidenced about a 27% increase in IP_3 accumulation, while the I26Q version represented and about a 32% increase.

Example 6
GPCR FUSION PROTEIN PREPARATION

The design of the constitutively activated GPCR-G protein fusion construct was accomplished as follows: both the 5' and 3' ends of the rat G protein Gsα (long form; Itoh, H. et al., 83 PNAS 3776 (1986)) were engineered to include a HindIII (5'-AAGCTT-3') sequence thereon. Following confirmation of the correct sequence (including the flanking HindIII sequences), the entire sequence was shuttled into pcDNA3.1(-) (Invitrogen, cat. no. V795-20) by subcloning using the HindIII restriction site of that vector. The correct orientation for the Gsα sequence was determined after subcloning into pcDNA3.1(-). The modified pcDNA3.1(-) containing the rat Gsα gene at HindIII sequence was then verified; this vector was now available as a "universal" Gsα protein vector. The pcDNA3.1(-) vector contains a variety of well-known restriction sites upstream of the HindIII site, thus beneficially providing the ability to insert, upstream of the Gs protein, the coding sequence of an endogenous, constitutively active GPCR. This same approach can be utilized to create other "universal" G protein vectors, and, of course, other commercially available or proprietary vectors known to the artisan can be utilized – the important criteria is that the sequence for the GPCR be upstream and in-frame with that of the G protein.

1. TSHR-Gsα Fusion Protein

a. Stable Cell Line Production for TSHR

Approximately 1.2 to 1.3 x 10^7 HEK-293 cells are plated on a 15cm tissue culture plate. Grown in DME High Glucose Medium containing ten percent fetal bovine serum and one percent sodium pyruvate, L-glutamine, and antibiotics. Twenty-four hours following plating of 293 cells to ~80% confluency, the cells are transfected using 12μg of DNA. The 12μg of DNA is combined with 60μL of lipofectamine and 2mL of DME High Glucose Medium without serum. The medium is aspirated from the plates and the cells are washed once with medium without serum. The DNA, lipofectamine, and medium mixture is added to the plate along with 10mL of medium without serum. Following incubation at 37°C for four to five hours, the medium is aspirated and 25ml of medium containing serum is added. Twenty-four hours following transfection, the medium is aspirated again, and fresh medium with serum is added. Forty-eight hours following transfection, the medium is aspirated and medium with serum is added containing geneticin (G418 drug) at a final concentration of 500 μg/mL. The transfected cells now undergo selection for positively transfected cells containing the G418 resistant gene. The medium is replaced every four to five days as selection occurs. During selection, cells are grown to create stable pools, or split for stable clonal selection.

b. TSHR(A623K) Fusion Protein

TSHR-Gsα Fusion Protein construct was then made as follows: primers were designed for both endogenous, constitutively activated and non-endogenous, constitutively activated TSHR were as follows:

5'-gatc[TCTAGA]ATGAGGCCGGCGGACTTGCTGC -3' (SEQ.ID.NO.:582; sense)

5'-ctag[GATATC]CGCAAAACCGTTTGCATATACTC -3' (SEQ.ID.NO.:583; antisense).

Nucleotides in lower caps are included as spacers just before the restriction sites between the endogenous TSHR and G protein. The sense and anti-sense primers included the restriction sites for XbaI and EcorV, respectively.

PCR was then utilized to secure the respective receptor sequences for fusion within the Gsα universal vector disclosed above, using the following protocol for each: 100ng cDNA for TSHR(A623K) was added to separate tubes containing 2 μL of each primer (sense and anti-sense), 3 μL of 10mM dNTPs, 10μL of 10XTaqPlus™ Precision buffer, 1 μL of TaqPlus™ Precision polymerase (Stratagene: #600211), and 80 μL of water. Reaction temperatures and cycle times for TSHR were as follows: the initial denaturing step was done at 94°C for five minutes, and a cycle of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for two minutes. A final extension time was done at 72°C for ten minutes. PCR product for was run on a 1% agarose gel and then purified (data not shown). The purified product was digested with XbaI and EcorV (New England Biolabs) and the desired inserts isolated, purified and ligated into the Gs universal vector at the respective restriction site. The positive clones were isolated following transformation and determined by restriction enzyme digest; expression using Hek-293 cells was accomplished following the protocol set forth *infra*. Each positive clone for TSHR: Gs–Fusion Protein was sequenced and made available for the direct identification of candidate compounds. (*See,* SEQ.ID.NO.:588 for nucleic acid sequence and SEQ.ID.NO.:589 for amino acid sequence).

Location of non-endogenous version of TSHR(A623K) is located upstream from the rat G protein Gsα (*i.e.,* from nucleotide 1 through 2,292; *see,* SEQ.ID.NO.:586 and amino acid residue 1 through 764; *see,* SEQ.ID.NO.:587). TSHR(A623K) can be linked directly to the G protein, or there can be spacer residues between the two. With respect to

TSHR, 24 amino acid residues (an equivalent of 72 nucleotides) were placed in between the non-endogenous GPCR and the start codon for the G protein Gsα. Therefore, the Gs protein is located at nucleotide 2,365 through 3,549 (*see*, SEQ.ID.NO.:586) and at amino acid residue 789 through 1,183 (*see*, SEQ.ID.NO.:587). Those skilled in the art are credited with the ability to select techniques for constructing a GPCR Fusion Protein where the G protein is fused to the 3' end of the GPCR of interest.

GPCR Fusion Protein was analyzed (to stabilize the GPCR while screening for candidate compounds, as shown in Example 6) and verified to be constitutively active utilizing the protocol found in Example 4(2). In Figure 5, TSHR(A623K)-Gαs:Fusion Protein evidenced about an 87% increase in cAMP when compared to the control vector (pCMV).

2. GPR24-Giα Fusion Protein

GPR24-Giα Fusion Protein construct was then made as follows: primers were designed for both endogenous, constitutively activated and non-endogenous, constitutively activated GPR24 were as follows:

5'-GTGAAGCTTGCCCGGGCAGGATGGACCTGG-3' (SEQ.ID.NO.:584; sense)

5'-ATCTAGAGGTGCCTTTGCTTTCTG-3' (SEQ.ID.NO.:585; anitsense).

The sense and anti-sense primers included the restriction sites for KB4 and XbaI, respectively.

PCR was then utilized to secure the respective receptor sequences for fusion within the Giα universal vector disclosed above, using the following protocol for each: 100ng cDNA for GPR24 was added to separate tubes containing 2 μL of each primer (sense and anti-sense), 3μL of 10mM dNTPs, 10μL of 10XTaqPlus™ Precision buffer, 1μL of TaqPlus™ Precision polymerase (Stratagene: #600211), and 80μL of water. Reaction

temperatures and cycle times for GPR24 were as follows: the initial denaturing step was done it 94°C for five minutes, and a cycle of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for two minutes. A final extension time was done at 72°C for ten minutes. PCR product for was run on a 1% agarose gel and then purified (data not shown). The purified product was digested with KB4 and XbaI (New England Biolabs) and the desired inserts will be isolated, purified and ligated into the Gi universal vector at the respective restriction site. The positive clones was isolated following transformation and determined by restriction enzyme digest; expression using Hek-293 cells was accomplished following the protocol set forth *infra*. Each positive clone for GPR24: Gi–Fusion Protein was sequenced and made available for the direct identification of candidate compounds. (*See*, SEQ.ID.NO.:590 for nucleic acid sequence and SEQ.ID.NO.:591 for amino acid sequence).

Endogenous version of GPR24 was fused upstream from the G protein Gi and is located at nucleotide 1 through 1,059 (*see,* SEE.ID.NO.:588) and amino acid residue 1 through 353 (*see,* SEQ.ID.NO.:589). With respect to GPR24, 2 amino acid residues (an equivalent of 6 nucleotides) were placed in between the endogenous (or non-endogenous) GPCR and the start codon for the G protein Giα. Therefore, the Gi protein is located at nucleotide 1,066 through 2,133 (*see*, SEQ.ID.NO.:588) and at amino acid residue 356 through 711 (*see*, SEQ.ID.NO.:589). Those skilled in the art are credited with the ability to select techniques for constructing a GPCR Fusion Protein where the G protein is fused to the 3' end of the GPCR of interest.

Although it is indicated above that Gi coupled receptors, such as GPR24, can be used in conjunction with a co-transfection approach, this is in the context of cAMP based assays and is predicated upon the effect of Gi on cAMP levels. However, for other types of assays, such as a GTP based assay, the co-transfection approach is not essential. Thus

for assays such as a GTP based assay, the GPCR Fusion Protein approach is preferred such that, with respect to a GTP based assay for GPR24, the GPR24:Gi Fusion Protein would be preferred.

Example 6
PROTOCOL: DIRECT IDENTIFICATION OF INVERSE AGONISTS AND AGONISTS USING [^{35}S]GTPγS

Although Endogenous GPCRs may be utilized for the direct identification of candidate compounds as, *e.g.*, inverse agonists, for reasons that are not altogether understood, intra-assay variation can become exacerbated. Preferably, then, a GPCR Fusion Protein, as disclosed above, can also be utilized with a non-endogenous, constitutively activated GPCR. We can determine that when such a protein is used, intra-assay variation appears to be substantially stabilized, whereby an effective signal-to-noise ratio is obtained. This has the beneficial result of allowing for a more robust identification of candidate compounds. Thus, it is preferred that for direct identification, a GPCR Fusion Protein be used and that when utilized, the following assay protocols be utilized.

1. Membrane Preparation

Membranes comprising the non-endogenous, constitutively active GPCR Fusion Protein of interest and for use in the direct identification of candidate compounds as inverse agonists, agonists or partial agonists are preferably prepared as follows:

a. Materials

"Membrane Scrape Buffer" is comprised of 20mM HEPES and 10mM EDTA, pH 7.4; "Membrane Wash Buffer" is comprised of 20 mM HEPES and 0.1 mM EDTA, pH 7.4; "Binding Buffer" is comprised of 20mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4.

b. Procedure

All materials will be kept on ice throughout the procedure. First, the media is aspirated from a confluent monolayer of cells, followed by rinse with 10ml cold PBS, followed by a aspiration. Thereafter, 5ml of Membrane Scrape Buffer will be added to scrape cells; this is followed by transfer of cellular extract into 50ml centrifuge tubes (centrifuged at 20,000 rpm for 17 minutes at 4°C). Thereafter, the supernatant is aspirated and the pellet is resuspended in 30ml Membrane Wash Buffer followed by centrifugation at 20,000 rpm for 17 minutes at 4°C. The supernatant will then be aspirated and the pellet resuspended in Binding Buffer. This is then homogenized using a Brinkman Polytron™ homogenizer (15-20 second bursts until the all material is in suspension). This is referred to herein as "Membrane Protein".

2. Bradford Protein Assay

Following the homogenization, protein concentration of the membranes will be determined using the Bradford Protein Assay (protein can be diluted to about 1.5mg/ml, aliquoted and frozen (-80°C) for later use; when frozen, protocol for use is as follows: on the day of the assay, frozen Membrane Protein is thawed at room temperature, followed by vortex and then homogenized with a Polytron at about 12 x 1,000 rpm for about 5-10 seconds; it is noted that for multiple preparations, the homogenizer should be thoroughly cleaned between homogenization of different preparations).

a. Materials

Binding Buffer (as per above); Bradford Dye Reagent; Bradford Protein Standard are utilized, following manufacturer instructions (Biorad, cat. no. 500-0006).

b. Procedure

Duplicate tubes will be prepared, one including the membrane, and one as a control "blank". Each contained 800 μL Binding Buffer. Thereafter, 10 μL of Bradford Protein Standard (1mg/ml) is added to each tube, and 10 μL of membrane Protein is then added to just one tube (not the blank). Thereafter, 200 μL of Bradford Dye Reagent is added to each tube, followed by vortex of each. After five (5) minutes, the tubes will be re-vortexed and the material therein is transferred to cuvettes. The cuvettes are then read using a CECIL 3041 spectrophotometer, at wavelength 595.

3. Direct Identification Assay

a. Materials

GDP Buffer consists of 37.5 ml Binding Buffer and 2mg GDP (Sigma, cat. no. G-7127), followed by a series of dilutions in Binding Buffer to obtain 0.2μM GDP (final concentration of GDP in each well was 0.1μM GDP); each well comprising a candidate compound, will have a final volume of 200 μL consisting of 100 μL GDP Buffer (final concentration, 0.1μM GDP), 50μL Membrane Protein in Binding Buffer, and 50 μL [^{35}S]GTPγS (0.6 nM) in Binding Buffer (2.5 μL [^{35}S]GTPγS per 10ml Binding Buffer).

b. Procedure

Candidate compounds are preferably screened using a 96-well plate format (these can be frozen at -80°C). Membrane Protein (or membranes with expression vector excluding the GPCR Fusion Protein, as control) will be homogenized briefly until in suspension. Protein concentration is then determined using the Bradford Protein Assay set forth above. Membrane Protein (and control) is then diluted to 0.25mg/ml in Binding Buffer (final assay concentration, 12.5 μg/well). Thereafter, 100 μL GDP Buffer will be added to each well of a Wallac Scintistrip™ (Wallac). A 5 μL pin-tool is then used to transfer 5 μL of a candidate compound into such well (*i.e.*, 5 μL in total assay volume of

200 μL is a 1:40 ratio such that the final screening concentration of the candidate compound is 10μM). Again, to avoid contamination, after each transfer step the pin tool should be rinsed in three reservoirs comprising water (1X), ethanol (1X) and water (2X) – excess liquid should be shaken from the tool after each rinse and dried with paper and kimwipes. Thereafter, 50 μL of Membrane Protein is added to each well (a control well comprising membranes without the GPCR Fusion Protein is also utilized), and pre-incubated for 5-10 minutes at room temperature. Thereafter, 50 μL of [^{35}S]GTPγS (0.6 nM) in Binding Buffer will be added to each well, followed by incubation on a shaker for 60 minutes at room temperature (again, in this example, plates were covered with foil). The assay is then stopped by spinning of the plates at 4000 RPM for 15 minutes at 22°C. The plates will then be aspirated with an 8 channel manifold and sealed with plate covers. The plates are then read on a Wallacc 1450 using setting "Prot. #37" (per manufacturer instructions).

Example 7

PROTOCOL: CONFIRMATION ASSAY

Using an independent assay approach to provide confirmation of a directly identified candidate compound as set forth above, it is preferred that a confirmation assay then be utilized. In this case, the preferred confirmation assay is a cyclase-based assay.

A modified Flash Plate™ Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) is preferably utilized for confirmation of candidate compounds directly identified as inverse agonists and agonists to non-endogenous, constitutively activated GPCR in accordance with the following protocol.

Transfected cells will be harvested approximately three days after transfection. Membranes are prepared by homogenization of suspended cells in buffer containing 20mM HEPES, pH 7.4 and 10mM $MgCl_2$. Homogenization is performed on ice using a Brinkman

Polytron™ for approximately 10 seconds. The resulting homogenate will be centrifuged at 49,000 X g for 15 minutes at 4°C. The resulting pellet is then resuspended in buffer containing 20mM HEPES, pH 7.4 and 0.1 mM EDTA, homogenized for 10 seconds, followed by centrifugation at 49,000 X g for 15 minutes at 4°C. The resulting pellet can be stored at -80°C until utilized. On the day of direct identification screening, the membrane pellet is slowly thawed at room temperature, resuspended in buffer containing 20mM HEPES, pH 7.4 and 10mM $MgCl_2$, to yield a final protein concentration of 0.60mg/ml (the resuspended membranes are placed on ice until use).

cAMP standards and Detection Buffer (comprising 2 μCi of tracer [^{125}I cAMP (100 μl)] to 11 ml Detection Buffer) will be prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer will be prepared fresh for screening and contained 20mM HEPES, pH 7.4, 10mM $MgCl_2$, 20mM phospocreatine (Sigma), 0.1 units/ml creatine phosphokinase (Sigma), 50 μM GTP (Sigma), and 0.2 mM ATP (Sigma); Assay Buffer can be stored on ice until utilized.

Candidate compounds identified as per above (if frozen, thawed at room temperature) will then be added, preferably, to 96-well plate wells (3 μl/well; 12μM final assay concentration), together with 40 μl Membrane Protein (30μg/well) and 50μl of Assay Buffer. This mixture is then incubated for 30 minutes at room temperature, with gentle shaking.

Following the incubation, 100μl of Detection Buffer is added to each well, followed by incubation for 2-24 hours. Plates are then counted in a Wallac MicroBeta™ plate reader using "Prot. #31" (as per manufacturer instructions).

Example 8

LIGAND-BASED CONFIRMATION ASSAY

Membranes will be prepared from transfected Hek-293 cells (*see* Example 3) by homogenization in 20 mM HEPES and 10 mM EDTA, pH 7.4 and centrifuged at 49,000 x g for 15 min. The pellet will be resuspended in 20 mM HEPES and 0.1 mM EDTA, pH 7.4, homogenized for 10 sec using Polytron homogenizer (Brinkman) at 5000 rpm and centrifuged at 49,000 x g for 15 min. The final pellet will be resuspended in 20 mM HEPES and 10 mM $MgCl_2$, pH 7.4, homogenized for 10 sec using Polytron homogenizer (Brinkman) at 5000 rpm.

Ligand-based confirmation assays will be performed in triplicate 200μl volumes in 96 well plates. Assay buffer (20 mM HEPES and 10 mM $MgCl_2$, pH 7.4) will be used to dilute membranes, tritiated inverse agonists and/or agonists and the receptor's endogenous ligand (used to define non-specific binding). Final assay concentrations will consist of 1nM of tritiated inverse agonist and/or agonist, 50μg membrane protein (comprising the receptor) and 100μm of endogenous ligand. Agonist assay will be incubated for 1 hr at 37° C, while inverse agonist assays are incubated for 1 hr at room temperature. Assays will terminate by rapid filtration onto Wallac Filtermat Type B with ice cold binding buffer using Skatron cell harvester. The radioactivity will be determined in a Wallac 1205 BetaPlate counter.

Again, this approach is used merely to understand the impact of the directly identified candidate compound on ligand binding. As those in the art will appreciate, it is possible that the directly identified candidate compounds may be allosteric modulators, (*i.e.,* compounds that affect the functional activity of the receptor but which do not inhibit the endogenous ligand from binding to the receptor. Allosteric modulators include inverse agonists, partial agonists and agonists.

References cited throughout this patent document, including co-pending and related patent applications, unless otherwise indicated, are fully incorporated herein by reference.

Modifications and extension of the disclosed inventions that are within the purview of the skilled artisan are encompassed within the above disclosure and the claims that follow.

Although a variety of expression vectors are available to those in the art, for purposes of utilization for both the endogenous and non-endogenous known GPCRs, it is most preferred that the vector utilized be pCMV. This vector was deposited with the American Type Culture Collection (ATCC) on October 13, 1998 (10801 University Blvd., Manassas, VA 20110-2209 USA) under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. The DNA was tested by the ATCC and determined to be. The ATCC has assigned the following deposit number to pCMV: ATCC #203351.